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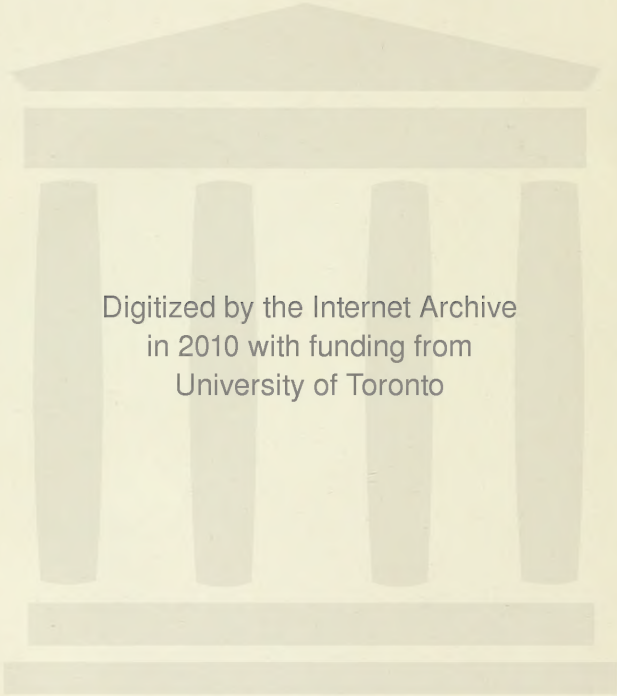
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ON THE GENERALIZATION OF TREPONEMA PALLIDUM IN THE RABBIT FOLLOWING LOCAL INOCULATION.

By LOUISE PEARCE and WADE H. BROWN.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

That a widespread dissemination of *Treponema pallidum* may be produced in the rabbit by local inoculation has been shown by the recovery of the organisms in isolated instances from the blood, lymph nodes or other organs as well as by the occasional occurrence of generalized lesions in infected animals. However, there is no evidence to show either the time or frequency with which this dissemination occurs or whether the organisms thus distributed over the body are capable of sustaining the infection in these animals.

With these questions in mind, a series of experiments was undertaken, the object of which was to determine the frequency of invasion of the regional lymphatics and the general circulation following inoculation in the scrotum or testicles and how soon a self-sustaining generalized infection might be established.

Time and Frequency of Invasion of Regional Lymphatics. –An examination was made of the inguinal lymph nodes in a series of 29 rabbits which had been inoculated by the introduction of a bit of infected tissue beneath the skin of the scrotum. The nodes were excised under ether anesthesia at intervals of from 61 days down to 48 hours after inoculation and the presence or absence of *Treponema pallidum* determined by dark field examination or by animal inoculation.

The first group of nodes studied included those showing well marked enlargement and induration and these gave positive results in all cases. Nodes were then taken 5 days after inoculation and after the lapse of only 48 hours. Positive results were again obtained in all cases.

Invasion of the Blood Stream.—A similar series of experiments was carried out to determine the time and frequency of blood stream invasion and something of the character of the blood stream infection with relation to processes of reaction in the primary lesions. With

a few exceptions, the animals used for these experiments were inoculated in the testicles. The mode of determining the presence of *Treponema pallidum* in the blood of infected animals was by bleeding from the heart, defibrinating and injecting 0.5 c.c. of blood into each testicle of 2 normal rabbits.

A total of 81 bleedings was made on a series of 37 rabbits at intervals of from 7 to 99 days after inoculation. The earlier bleedings were all spaced with reference to some phase of the testicular infection and from these it was found that organisms could be recovered from the circulating blood from the time an infection could first be detected clinically (12 to 14 days) until regression of the primary lesions took place. The number or the virulence of the organisms as indicated by the incubation period and the constancy of infection in subinoculated animals varied, however, according to the stage of development and the state of the reaction in the primary lesions.

A small series of animals was then bled arbitrarily one week after inoculation and it was found that even as early as this, the number of organisms in the circulating blood was sufficiently great for each 0.5 c.c. of blood to constitute an infecting dose. In view of these facts, there seemed to be no immediate object in further reducing the time limits.

The Establishment of a (True) Generalized Infection.—When it had been shown that *Treponema pallidum* appeared to be widely distributed through the body within a very short time after inoculation, it was considered necessary to determine whether a true generalized infection had been established in these animals or whether the organisms proved viable only because they were transferred to such a favorable medium as the testicles of normal rabbits. For this purpose, 10 rabbits were inoculated in the right scrotum only (using implants), and 48 hours later, the entire right scrotum and testicle were amputated under ether anesthesia. In spite of the complete removal of a wide zone surrounding the area of inoculation, 9 of the 10 rabbits showed well-marked infections by the end of the seventh week and the tenth developed lesions at the end of $2\frac{1}{2}$ months after inoculation.

CONCLUSIONS.

These experiments show that following a local inoculation of well adapted strains of *Treponema pallidum*, there is an immediate invasion of the tissues of the animal and that within a very short time, organisms may be recovered from both the regional lymphatics and the circulating blood. They also show that the blood stream infection tends to pursue a course parallel with that of the primary lesions. Finally it was shown that within 48 hours or less, a true generalized infection had been established which was capable of maintaining the infection in the animal independent of that at the site of inoculation.

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ETIOLOGY OF YELLOW FEVER.

XII. Chemotherapy versus Serotherapy in Experimental Infection with Leptospira icteroides.

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(Received for publication, April 3, 1920.)

The work of Ehrlich and Hata[1] on the therapeutic effects of arsenical organic compounds in various infections caused by spirochetal or protozoan organisms has led to considerable investigation by later workers, notably Jacobs, Heidelberger, Amoss, and Bull[2–5] and Pearce and Brown,[6] of the extent to which this kind of curative method is applicable. The present experiments were undertaken, not as research in chemotherapy, but merely as part of a routine study of experimental infection in guinea pigs with *Leptospira icteroides*, derived from certain cases of yellow fever.[7] It was of particular interest to ascertain how *Leptospira icteroides* would behave toward the two widely employed chemotherapeutic agents, salvarsan and neosalvarsan, and what difference, if any, there is between its behavior and that of the inciting agent of infectious jaundice in this respect, for the latter has been extensively studied by Inada, Ido, and their collaborators,[8] as well as

[1] Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.

[2] Jacobs, W. A., *J. Exp. Med.*, 1916, xxiii, 563.

[3] Jacobs, W. A., Heidelberger, M., and Amoss, H. L., *J. Exp. Med.*, 1916, xxiii, 569.

[4] Jacobs, W. A., Heidelberger, M., and Bull, C. G., *J. Exp. Med.*, 1916, xxiii, 577.

[5] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1915, xx, 513, 659, 685; xxi, 103, 145, 403, 439, 455, 465.

[6] Pearce, L., and Brown, W. H., *J. Exp. Med.*, 1918, xxviii, 109.

[7] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 1, 9, 13, 87, 95, 401; 1920, xxxi, 135, 159.

[8] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377. Kaneko, R., and Okuda, K., *Verhandl. japan. path. Ges.*, 1916, vi, 49.

by European investigators, especially Uhlenhuth and Fromme.[9] A large number of experiments with salvarsan and neosalvarsan, atoxyl, and arsacetin against *Leptospira icterohæmorrhagiæ* was also carried out in 1917, with negative results.[10] Being regarded by some as a spirochete, *Leptospira icterohæmorrhagiæ* was expected to yield to chemotherapy by means of salvarsan or its derivative, but Inada and Ido and others soon found that neither salvarsan nor neosalvarsan had any definite therapeutic value in infections with this organism. In this respect, at least, the organism discovered by Inada and Ido in infectious jaundice did not behave like the other pathogenic spirochetes, and this characteristic may be regarded as giving further support to the opinion that the leptospira group of organisms forms a type of its own and differs from the other pathogenic genera of Spirochætoidea. The experiments here reported concern the behavior of *Leptospira icteroides* toward the arsenical compounds, not only in the animal body, but also *in vitro*, and the effect upon the organism of salvarsanized serum. From the practical standpoint it seemed advisable to include also a brief comparison of the action of the arsenical preparations and that of anti-*icteroides* immune serum upon *Leptospira icteroides* in experimental infection and *in vitro*.

Effect of Salvarsan and Neosalvarsan upon the Course of Experimental Infection of Guinea Pigs Inoculated with Leptospira icteroides.

Several series of experiments were performed in order to ascertain whether administration of salvarsan or neosalvarsan simultaneously with the inoculation or shortly afterwards would in any way influence the development of the experimental leptospiral infection in guinea pigs. If the virulence of *Leptospira icteroides* for this animal were constant a few series of experiments only would have been sufficient to determine the point, but owing to the variable character of the pathogenicity of the organism for individual guinea pigs it was necessary to repeat similar experiments. In some series control animals survived or escaped infection, hence the interpretation of the effect of the drugs was rendered inconclusive. In the earlier experiments

[9] Uhlenhuth and Fromme, *Z. Immunitätsforsch., Orig.*, 1916, xxv, 418.
[10] Noguchi, H., unpublished results.

the amounts of infecting material were near a single lethal dose, even subminimum lethal doses being used; *i.e.*, a quantity capable of producing in the majority of instances a mild infection with recovery. The mode of experiment was practically the same in all the series. In one instance an unneutralized solution of salvarsan was employed, otherwise salvarsan has been used as an alkaline solution. The injection of the infecting material was intraperitoneal and that of the drugs subcutaneous.

TABLE I.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug

November 27, 1919. The amount of infecting material used was 0.5 cc.

Amount of arsaminol natrium	Course of disease.	Result.	Remarks.
gm.			
0.03	Mild; 6 to 8 days.	Recovered.	Died in 15 days; no jaundice.
0.02	Moderate (+); 6 to 11 days.	"	No jaundice.
0.01	Severe (++++); 6 to 11 days.	"	Died in 13 days; jaundice fading.
0.005	Mild; 7 to 9 days.	"	No jaundice.
0.0025	Severe (++++); 6 to 11 days.	"	Killed in 11 days; typical lesions.
0.001	Died in 6 days.	Typical.	
0.0005	" " 9 "	"	
Control.	Severe (+++); killed in 6 days.	"	
"	Moderate (++); 7 to 8 days.	Recovered.	
"	Mild (±); 7 days.	"	

Series 1.—November 27, 1919. The infecting material consisted of a mixture of citrate blood (showing the leptospiras) of a guinea pig experimentally infected with Guayaquil Strain 1 and a rich culture of the same strain. Ten guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of the mixture, and all but three (controls) then received subcutaneously a solution of arsaminolnatrium (a Japanese preparation of neosalvarsan), the quantities given ranging from 0.03 to 0.0005 gm. (Table I).

The amount of infecting material used in this series was near a single minimum lethal dose, as shown in the control animals and also in those which received the smallest amounts of the drug. It is noteworthy that in the three guinea pigs which received 0.03, 0.02,

and 0.005 gm. of the drug, respectively, there was no jaundice at any period of the infection.

Series 2.—December 3, 1919. The infecting material consisted of a mixture of 1 cc. each of cultures of Guayaquil Strains 1 and 5, and 7 cc. of heart's blood from a guinea pig infected typically with Strain 1. 0.5 cc. of the mixture was inoculated intraperitoneally into each of sixteen guinea pigs. One group of eight animals was then inoculated subcutaneously with an unneutralized solution of salvarsan and the other group of eight with the solution of neosalvarsan in doses

TABLE II.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

December 3, 1919. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used.	Course of disease.
	Salvarsan (acid solution).	Neosalvarsan.		
gm.			*cc.*	
0.03	Mild; recovery.	Mild; recovery.	1.0	Severe (++++); recovery.
0.02	No symptoms.	Severe (+++); recovery.	0.5	Mild; recovery.
			0.2	Moderate (++); recovery.
0.01	" "	Mild; recovery.		
0.005	" "	No symptoms.	0.1	No symptoms.
0.002	Died in 12 days; typical.	" "		
0.001	No symptoms.	" "		
0.0005	" "	Died in 13 days; typical.		
0.0002	" "	Severe (+++); recovery.		

varying from 0.03 to 0.0002 gm. There were four control guinea pigs which received varying doses of the infecting material and were not treated with the drug (Table II).

The results of these experiments suggest a possible slight protective effect of the drugs in some individuals, although, from the mildness of the infection in the control animals the survival of those individuals may also be explained on the basis of a natural resistance to *Lepto-*

spira icteroides. It is of interest to note that more guinea pigs among those treated with salvarsan escaped the infection than among those treated with neosalvarsan. The indecisive character of the experiments made necessary another series with multiple minimum lethal doses. In Series 3 at least 50 minimum lethal doses were used for each guinea pig.

Series 3.—March 13, 1920. The infecting material consisted of a mixture of 5 cc. of a culture of Guayaquil Strain 1, 5 cc. of kidney emulsion, and 5 cc. of citrated heart's blood from a guinea pig inoculated with the same strain 4 days previously. In order to produce a fatal infection in guinea pigs 0.5 cc. of the mixture was inoculated intraperitoneally into each animal. As the protocol shows, 0.01 cc. of this material killed a control animal in 7 days with typical symptoms, and a much smaller quantity would have been sufficient to cause a fatal infection, although the minimum lethal dose was not determined in the present series of experiments.

Salvarsan was dissolved in sterile distilled water and a 0.1 N sodium hydroxide solution was gradually added until the precipitate first formed was completely redissolved. The final concentration of the drug was made 0.1 gm. per 10 cc. (stock solution). Neosalvarsan was dissolved in sterile distilled water in the ratio of 0.1 gm. per 10 cc. (stock solution). Further dilutions were made with 0.9 per cent salt solution.

The animals were inoculated with 0.5 cc. of the infecting material intraperitoneally, and within about 30 minutes varying amounts of salvarsan and neosalvarsan solution were injected subcutaneously. The amounts of the drug were 0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, and 0.003 gm. for guinea pigs of 350 gm., and each dose was contained in from 0.5 to 3 cc. of fluid, according to convenience in measurement. Table III gives the results.

Series 4.—March 18, 1920. Although it was still too early to know the results of the series of March 13, another series was begun on March 18 in which larger doses (0.01, 0.02, and 0.03 gm.) of salvarsan and neosalvarsan were used. The solutions of the drugs were freshly prepared from another set of ampules, and the infecting material consisted of a guinea pig kidney emulsion of Guayaquil Strain 1 of *Leptospira icteroides*. Other technical details were the same as in the previous experiments. Table IV gives the results.

The experiments of Series 3 (March 13) show that guinea pigs receiving at least 50 minimum lethal doses of a strain of *Leptospira icteroides* intraperitoneally and varying quantities of salvarsan or neosalvarsan subcutaneously within 30 minutes from the time of inoculation of *icteroides* undergo a typical course of leptospira infection, resulting in the majority of instances in death within a

period the variations of which may be considered usual in such a series of experiments. With salvarsan there were two instances of recovery after a severe infection coinciding with elevated doses of the drug (0.001 and 0.003 gm.), while in animals receiving less of the

TABLE III.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 13, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug	Course of disease.		Test for virulence of infecting material used	Course of disease.
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.00005	Died in 5 days; typical.	Recovery after a mild infection, with definite jaundice from Mar. 20–24, 1920.	0.5	Died in 6 days; typical.
			0.1	Died in 7 days; typical.
0.0001	" " 8 " "	Died in 5 days; typical.	0.01	Died in 7 days; typical.
0.0002	" " 5 " "	" " 6 " "		
0.0005	" " 6 " "	" " 7 " "		
0.001	Recovery after a typical infection, with intense jaundice from Mar. 19–25, 1920.	" " 9 " "		
0.002	Died in 6 days; typical.	Recovery after a mild infection, with jaundice from Mar. 19–23, 1920.		
0.003	Recovery after a typical infection lasting from Mar. 18–20, 1920.	Recovery after a severe infection, with intense jaundice from Mar. 18–23, 1920.		

drug death occurred in two instances 2 days earlier than in controls, a fact worthy of notice. With neosalvarsan the number of recovered animals was three, two with the largest doses of the drug (0.002 and 0.003 gm.) and one with the minutest dose employed (0.00005 gm.).

All the rest died in from 5 to 9 days with typical symptoms. The results both with salvarsan and with neosalvarsan suggest that these arsenical compounds, when employed in certain quantities, may somewhat modify the severity of the infection and occasionally save a guinea pig from death, although failing to suppress the infection completely.

The results of the experiments of Series 4 (March 18) were less favorable than those of Series 3. Here, of the two controls one died in 12 days and the other recovered after a definite infection. Two

TABLE IV.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Drug.

March 18, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Amount of drug.	Course of disease.		Test for virulence of infecting material used	Course of disease
	Salvarsan.	Neosalvarsan.		
gm.			*cc.*	
0.01	Died in 11 days; typical.	Died in 12 days; typical.	0.5	Died in 12 days; typical.
0.02	Had fever but no jaundice; recovery.	" " 12 " "	0.5	Recovery after a definite infection, with intense jaundice from Mar. 30–31, 1920.
0.03	Died in 11 days; typical.	Had fever but no jaundice; recovery.		

of the three guinea pigs treated, either with salvarsan or neosalvarsan, died in from 11 to 12 days, and the one animal which survived in each set had fever indicative of an abortive leptospira infection.

From the therapeutic standpoint neither salvarsan nor neosalvarsan is of any value in experimental infections of guinea pigs with *Leptospira icteroides*. In contrast, it may be interesting to include here a protocol illustrating the highly specific protective value of an anti-*icteroides* serum (horse). The amount of the immune serum required to protect a guinea pig from an infection with *Leptospira icteroides* is exceedingly minute.

Contrasted Effect of Anti-icteroides Immune Serum in Vivo.

March 11, 1920. The experiments with the immune serum were undertaken with the same strain of *Leptospira icteroides* (Guayaquil No. 1) that was used in the chemotherapeutic experiments already described. The material was a mixture of the emulsions of kidney and liver from a guinea pig which was showing early symptoms of the leptospira infection. As the protocol shows, the minimum lethal dose of this emulsion was such that 1 cc. of a 1:10,000 dilution of it killed a guinea pig with typical symptoms in 12 days, and the same quantity of a 1:1,000 or 1:100 in 10 days. 0.5 cc. of the original emulsion killed a guinea pig in 8 days.

TABLE V.

Inoculation of Infecting Material into Guinea Pigs Followed by Injection of Anti-icteroides Serum.

March 11, 1920. The amount of infecting material used for the treated animals was 0.5 cc.

Treated animals.			Untreated controls.	
Anti-*icteroides* immune serum.		Course of disease.	Test for virulence of infecting material used.	Course of disease.
Amount.	Dilution.			
cc.			*cc.*	
1	1:100,000	Recovery after a mild infection.	0.5	Died in 8 days; typical.
			0.1	Recovery after a severe infection (exceptional resistance).
1	1:10,000	No symptoms.		
1	1:1,000	" " (suspicion of trace of jaundice on Mar. 20, which had disappeared the following morning).	0.01	Died in 10 days; typical.
			0.001	" " 10 " "
			0.0001	" " 12 " "
			0.00001	No symptoms.
1	1:100	No symptoms.	0.000001	" "
1	1:10	" "		

For infecting guinea pigs to be treated with anti-*icteroides* serum, 0.5 cc. (about 5,000 minimum lethal doses) of the same emulsion was intraperitoneally injected. The different amounts of the anti-*icteroides* serum (collected from Horse 2 on February 25, 1920) were then injected, also intraperitoneally, within half an hour after the inoculation. The amounts of immune serum were 0.00001, 0.0001, 0.001, 0.01, and 0.1 cc. Table V is a record of the results.

According to a conservative estimate, therefore, the power of the immune serum is such that 1 cc. of a 1:10,000 dilution prevented an infection when the dose of the infecting material was 5,000 minimum

lethal doses. In other words, 1 cc. of the serum had the power to protect a guinea pig of 350 gm. body weight against 50,000,000 (10,000 × 5,000) minimum lethal doses when administered intraperitoneally 30 minutes after intraperitoneal inoculation. The specific protective property of the immune serum is indisputably highly efficacious as compared with salvarsan or neosalvarsan, the value of which is at least doubtful. A point of considerable importance is that in certain guinea pigs receiving small quantities of salvarsan and neosalvarsan the period before death seemed to be shortened by 2 days (5 days), as compared with the average period in untreated control animals (7 days). It may be that the predilection of arsenic compounds for the renal tissues had a definite predisposing effect, due to chemical injury, upon this organ, which *Leptospira icteroides* also attacks particularly.

Direct Action of Salvarsan and Neosalvarsan upon Leptospira icteroides.

Ehrlich makes a special point of having found, in his quest for chemotherapeutic agents, that a preparation whose destructive power on a microbic organism is great *in vitro* may have no, or only a slight antagonistic effect when introduced into the animal body, or the relation between the effect manifested *in vitro* and *in vivo* may be the reverse. Ehrlich's efforts to find a chemotherapeutic preparation were principally directed toward its effect *in vivo*, since the pathogenic parasites with which he was working belonged to the class of protozoa or a class closely allied to it, and there were no virulent cultures on hand to be tested *in vitro* as well as *in vivo*. Certain arsenic compounds elaborated by Ehrlich and his coworkers displayed a highly sterilizing effect on various spirochetoid organisms when introduced into the animal body infected with them, while the direct effect of these preparations upon the same organisms *in vitro* was almost nil. Ehrlich interpreted the highly parasiticidal properties of these compounds as due to a certain modification (reduction) in the animal body of substances otherwise comparatively inert. Salvarsan and *Treponema pallidum* constitute a good example in point.

Leptospira icteroides is resistant to saponin,[11] a property which alone would serve to differentiate it from certain other groups of spiro-

[11] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

chetoid organisms (*Treponema* and *Spironema*). The fact that *in vivo* the leptospiras were scarcely influenced by the introduction of salvarsan or neosalvarsan constituted another point of dissimilarity between the leptospira and other spirochetal parasites. Hence the effect of salvarsan or neosalvarsan upon the leptospira from yellow fever cases *in vitro* presented a further interesting subject for study. Several experiments were also performed to determine the behavior of blood serum derived from a rabbit which had been injected intravenously 1 hour previously with salvarsan or neosalvarsan in considerable quantities; that is, a study was made of the effect of salvarsanized serum, as well as of drug solutions, upon *icteroides in vitro*.

TABLE VI.

Addition of Drug to Culture of Leptospira icteroides.

November 26, 1919.

Final concentration of arsaminolnatrium.*	Result.
1:2,000	All inactive and most have disappeared.
1:6,000	" " " some degenerated.
1:20,000	" "
1:60,000	" " thinner, and smoother.
1:200,000	" "
1:600,000	For the most part inactive.
1:2,000,000	" " " " " " some still active.
Water (control).	" " " " active, a few inactive.

* The concentration resulting from the mixture of the drug solutions with the culture is given.

To each of several tubes containing 1 cc. of a culture of Guayaquil Strain 6 was added 1 cc. of different dilutions of salvarsan, neosalvarsan, or arsaminolnatrium. The mixtures were allowed to stand at room temperature and examined after 2 or 2½ hours, and again after 24 or 48 hours. As Tables VI and VII show, sufficient quantities of the drugs rendered the leptospiras immotile, and brought about general disintegration. The highest dilution of any of the drugs which still killed the organism was about 1:200,000.

In testing the leptospiricidal strength of the solutions of salvarsan or neosalvarsan, it was important to take note of the reactions of the solutions. Salvarsan, according to the usual practice, is first treated with sodium hydroxide solution until completely precipitated out

at the point of neutral reaction. In order to obtain a clear solution it is necessary to add more alkali until the precipitate completely dissolves. At this point the solution is no longer slightly, but intensely alkaline. By reducing the alkalinity with hydrochloric acid to a reaction of about pH 8 a bulky precipitate is once more formed, and at pH 7 the entire substance flocculates out of the solution. In making a microbicidal titration a clear alkaline solution was used in ascending dilutions to eliminate the destructive effect of the reaction alone. It was found that the 1:1,000 dilution was still too strongly

TABLE VII.

Addition of Drugs to Culture of Leptospira icteroides.

December 3, 1919.

Final concentration of the drug.	Salvarsan.		Neosalvarsan.	
	2½ hrs.	24 hrs.	2½ hrs.	24 hrs.
1:200	Precipitate (?).	Precipitate heavy.	All inactive.	All immobilized.
1:2,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:20,000	Some active; precipitate.	All immobile; precipitate.	" "	" "
1:200,000	For the most part active.	All immobile.	" "	" "
1:2,000,000	For the most part active.	For the most part immobile; few active.	For the most part active.	Few active.
Saline (control).	All active.	All active.	All active.	For the most part active.

alkaline to be used (far beyond pH 8), the leptospiras dying rapidly in such a solution, while dilutions higher than 1:10,000 had a reaction which was practically that of the diluent (saline solution), pH 7. Neosalvarsan dissolved in distilled water in 1:100 dilution is a clear amber-yellow and shows to phenol red an intense red color; in diluting to 1:1,000 with saline solution its reaction approaches pH 7.2; in 1:10,000 dilution it is no longer perceptible to phenol red. In actual experiments, however, 1 cc. of each of the dilutions of the drugs was mixed with 1 cc. of a rich culture of one of the *icteroides* strains which

had a reaction of pH 7.4. This mixing brought down the pH value of the lower dilutions and raised that of the higher. The reactions between pH 7 and pH 7.8 are well borne by *icteroides*, the optimum being near pH 7.2 to 7.4. A reaction beyond pH 8 or below pH 6.6 is unsuitable for the existence of the organism. The devitalizing action of salvarsan and neosalvarsan, even in optimum reactions, is rather slow, a contact of many hours being required before death ensues, as the protocol shows.

TABLE VIII.

Addition of Drugs to Culture of Leptospira icteroides.

March 18, 1920.

Final concentration of the drug.	Salvarsan.			Neosalvarsan.		
	Reaction.	After 15 min.	After 18 hrs.	Reaction.	After 15 min.	After 18 hrs.
1:200	Intensely alkaline.	Dead.		Slightly over pH 8.	Active.	Dead.
1:2,000	Intensely alkaline.	"		pH 7.2	"	"
1:20,000	pH 7.2	Active.	Dead.	pH 7.2	"	"
1:200,000	pH 7.2	"	"	pH 7.2	"	"
1:2,000,000	pH 7.2	"	" (?).	pH 7.2	"	Active.*
Saline control (no drug).	pH 7.2	"	Active.	pH 7.2	"	"

* All found dead after 96 hours; the control tube was lost through contamination.

To each of several tubes containing 1 cc. of the same culture of *icteroides*, Guayaquil Strain 5 (pH 7.4), was added 1 cc. of several different dilutions of drugs, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 of alkalinized salvarsan solution and neosalvarsan. The mixtures were allowed to stand at 26°C. and the contents examined under the dark-field microscope after 15 minutes and again after 18 hours. The results are recorded in Table VIII.

It is evident that *Leptospira icteroides* is highly sensitive to the action of salvarsan and neosalvarsan, but their action is comparatively slow, requiring many hours contact. The effects of the drugs in a culture medium are found to be approximately the same as in the case of direct mixing of culture and solutions.

Various dilutions of the drugs were added to the usual medium (rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.). The culture used was Guayaquil Strain 1, and the tubes were allowed to stand for 5 days at room temperature (26°C.). A medium containing salvarsan or neosalvarsan in a ratio of more than 1:200,000 was found to be unsuitable for the growth of the organism (Table IX). No attempt was made to determine quantitatively the exact leptospiricidal titers of the two drugs.

TABLE IX.

*Addition of Drugs to Culture Media.**

March 6, 1920.

Final concentration of the drug.	Salvarsan.	Neosalvarsan.
1:600	−	−
1:1,800	−	−
1:6,000	−	−
1:18,000	−	−
1:60,000	−	−
1:180,000	−	−
1:600,000	+	<+
1:1,800,000	+	+
1:6,000,000	+	+
Control.	+	+
"	+	+

* The medium consisted of rabbit serum, 25 per cent, agar, 0.3 per cent, total volume, 6 cc.

The changes in the color of salvarsan, when observed after 12 days standing, were noticeable in the tubes containing dilutions from 1:100,000 up, while with neosalvarsan the changes were not found in dilutions below 1:10,000.

Effect of Salvarsanized and Neosalvarsanized Serum.

The problem here was to determine the effect of the living body upon the drugs when the latter were introduced into the blood circulation. It has been assumed that in the animal or human body they are converted into highly spirocheticidal substances, as is said to be the case with respect to the organisms of syphilis, yaws, and relapsing fevers. It has already been shown that these drugs are practically without effect *in vivo* upon the course of the *icteroides* infection, and that they are highly destructive to the organism *in vitro*. It was this discrepancy which suggested the study of the salvarsanized serum.

The mode of experiment was to inject a rabbit intravenously with salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight, the blood being drawn 1 hour after the time of injection. Two rabbits, weighing 1,500 and 2,000 gm. respectively, were injected on March 18, 1920, intravenously, one with salvarsan (alkaline), the other with neosalvarsan. After 1 hour they were killed for the blood. The clear serums were collected the next day and used in the active state in order to determine whether they were in any way different from a normal rabbit serum when mixed *in vitro* with the same quantity (1 cc. in each case) of a rich culture of Guayaquil Strain 5. The mixtures were kept in a thermostat at 28°C. during the period of observation. Table X gives the results.

The experiments demonstrated clearly the difference between the normal and the salvarsanized rabbit serums. All the leptospiras mixed with the latter died within 72 hours, while those in normal rabbit

TABLE X.

Effect of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serum in Vitro.

March 18, 1920.

Duration of contact.	Salvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized rabbit serum, 1 cc., plus culture, 1 cc.	Normal rabbit serum, 1 cc., plus cultue, 1 cc.
hrs.			
1	All active.	All active.	All active.
18	For the most part motile, but sluggish.	For the most part sluggish.	" "
48	No observation.	No observation.	No observation.
72	All dead and degenerated.	All dead and degenerated.	All active and multiplying.

serum steadily multiplied. It may be mentioned that the reactions of the drugged serums as well as the reaction of the normal serum were pH 7.4, hence the question of hydrogen ion concentration does not enter into the present comparative study. At the end of 18 hours the organisms were already less active in the drugged serums than in the normal serum.

It occurred to me that the cause of this slow leptospiricidal action of the salvarsanized and neosalvarsanized serums might be due to a gradual development of injurious substances by slow oxidation of the drugs. If this were the case we should find in these tubes a powerful and rapidly acting leptospiricidal substance at the end of 72 hours of exposure to the same experimental conditions. Two different experiments were carried out to ascertain this point.

In the first experiment 0.5 cc. of a rich culture of the Merida strain[12] of *icteroides* was added to each of the three tubes containing salvarsanized, neosalvarsanized, and normal serum respectively. The results were similar to those observed with the Strain 5 culture; that is, there was no effect upon the organisms during the 1st hour; they were all active at the end of that time. After 24 hours the leptospiras in the salvarsanized serum were all dead, but there were many active survivors in the neosalvarsanized serum. After 48 hours, however, they were for the most part dead in the neosalvarsanized serum, but all were active in the tube containing the normal serum. In the second experiment three tubes containing 1 cc. of each of the serums were placed at 28°C. for 72 hours, then 1 cc. of the rich Merida culture was added to each. The mixtures were again put at 28°C. for the period of observation. Table XI gives the results of this experiment.

TABLE XI.

*Effect on Leptospira icteroides (Merida Culture) of Salvarsanized, Neosalvarsanized, and Normal Rabbit Serums after the Serums Had Stood for 72 Hours in the Incubator at 28°C.**

Experiment of Mar. 22, 1920.	Salvarsanized serum, 1 cc., plus culture, 1 cc.	Neosalvarsanized serum, 1 cc., plus culture, 1 cc.	Normal serum, 1 cc., plus culture, 1 cc.
After 1 hr.	All active.	All active.	All active.
" 18 hrs.	Some active.	Many active.	" "
" 48 "	All dead.	All dead.	" "

* There was a slight shift of pH value (to pH 7.8) in these serums on standing, but they were brought back to pH 7.6 by the addition of the culture.

These two series of experiments indicate that a certain antagonistic substance seems to have developed in the tubes containing the salvarsanized and neosalvarsanized serums during the period of 72 hours at 28°C., as shown by an earlier death and degeneration of the leptospiras than was the case with the fresh samples of these serums. The difference was especially definite in the effects observed at the end of 18 hours with the first series. On the other hand, in no instance was there any rapid immobilization or destruction of the organisms, the drugs having exerted no perceptible effect upon them even after an hour's contact. At all events, no rapidly leptospiricidal substances could be demonstrated in the salvarsanized or neosalvarsanized rabbit serum after exposure to the air for 72 hours at 28°C. The death of the leptospiras in these drugged serums was slow but certain.

[12] This strain was isolated from a case of yellow fever in Merida, Mexico, and will be described in a later paper (Noguchi, H., and Kligler, I. J., *J. Exp. Med.*, 1920, xxxii, in press).

The question arises as to the form in which salvarsan or neosalvarsan existed in the blood serum of these two rabbits. This we do not know, but whatever its state, its ultimate concentration must correspond at most with a dilution of 1:20,000; that is, on the assumption that 0.05 gm. of the compound had diffused out in a space of 1,000 cc. In reality, the volume representing 1 kilo of body weight of the rabbit must be considerably smaller than 1,000 cc., hence the concentration of the drugs in the serum must have been stronger than 1:20,000. Other experiments (Table VII) showed that salvarsan or neosalvarsan added directly to a rich culture kills the latter within 24 hours; that is, in less time than that required with the salvarsanized or neosalvarsanized serum, which was at least 48 hours. Perhaps the arsenic compounds had undergone a modification in the animal body which converted them into substances operating much more slowly. As the animals did not urinate after the injection of the drugs up to the time of collecting the serum the slowness of action cannot be explained by elimination of the drug through the urine. Moreover, a dilution of 1:200,000 of the drugs when added directly to a culture caused the death of the latter in 18 hours. To summarize, then, serum drawn from rabbits at the end of 1 hour from the time of an intravenous injection of salvarsan or neosalvarsan in a ratio of 0.05 gm. per kilo of body weight possesses a slowly acting leptospiricidal property, which does not seem to be much increased in respect to rate of action by an exposure to the air for a period of 72 hours at 28°C.

Contrasted Effect of Anti-icteroides Immune Serum in Vitro.

Comparison has already been made of the chemotherapeutic value of salvarsan and neosalvarsan and the serotherapeutic value of anti-*icteroides* immune horse serum, showing the comparative inefficacy of these drugs and the highly potent specific protective property of the serum. Since the leptospiricidal power of the drugs was considerable *in vitro*, in sharp contrast with their lack of perceptible protective action *in vivo*, a similar comparison of the action *in vitro* of the immune serum and that of the drugs was of practical interest.

The same immune serum which was used in the experiments recorded in Table V was mixed with 1 cc. of a rich culture of Guayaquil Strain 5, the object being

to determine how high a dilution of the serum still had leptospiricidal power in the test-tube. The maximum dilution which caused complete immobilization and subsequent degeneration within 1 hour was 1:20, while a 1:200 dilution caused considerable but incomplete agglutination and degeneration within 18 hours. No effect whatever was perceptible in a mixture containing the serum in a dilution of 1:1,000 or less. The results of the experiment are recorded in Table XII.

When we place side by side this low titer *in vitro* of the immune serum (1 cc. of a 1:100 dilution to 1 cc. of culture) and its protective titer *in vivo* (1 cc. of a 1:10,000 dilution to 0.5 cc. of culture, or 5,000 minimum lethal doses), it is easy to conceive at once how completely

TABLE XII.

Effect of Anti-icteroides Immune Serum upon Leptospira icteroides in Vitro.

March 19, 1920.

Anti-*icteroides* immune serum.		After 1 hr.	After 18 hrs.
Amount.	Dilution.		
cc.			
1	1:10 (final dilution 1:20).	All dead.	All degenerated.
1	1:100 (" " 1:200).	For the most part active.	For the most part agglutinated and degenerated, but some still motile.
1	1:1,000 (" " 1:2,000).	All active.	All active.
1	1:10,000 (" " 1:20,000).	" "	" "
1	1:100,000 (" " 1:200,000).	" "	" "
1	Saline control.	" "	" "

reverse is the relation that exists between the behavior of the immune serum on the one hand and that of salvarsan and neosalvarsan on the other toward *Leptospira icteroides in vitro* and *in vivo*.

SUMMARY AND CONCLUSIONS.

In several series of experiments guinea pigs were variously infected with different amounts of *Leptospira icteroides*, either in the form of culture, organ emulsion from infected guinea pigs, or a mixture of both. The infecting materials were of different grades of virulence; in some series the amount given was near a single lethal dose, in others a subminimum lethal dose was given, *i.e.* causing mild infection

with recovery in the majority of animals, and in still others the animals were injected with at least 50 minimum lethal doses of a mixture of a culture and a highly virulent organ emulsion from a guinea pig. The animals were inoculated intraperitoneally, and within about 30 minutes each was injected subcutaneously with a different amount of salvarsan or neosalvarsan. The amounts injected were in most series 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, and 0.03 gm. per 350 to 450 gm. of body weight, and in one series, in addition to this dosage, 0.00005, 0.0001, and 0.0002 gm. were also tried.

Among the guinea pigs treated either with salvarsan or with neosalvarsan there were more recoveries than among the controls, but they were not in strict proportion to the amounts of the drugs injected. In the experiments with 50 minimum lethal doses of the infecting material there were several recoveries among those which received 0.001 to 0.002 to 0.003 gm., but all passed through a typical infection with all its symptoms. It is extremely doubtful, therefore, whether salvarsan or neosalvarsan mitigated the severity of the infection. The fact is noteworthy that in the same series of experiments the guinea pigs receiving 0.00005 and 0.0001 gm., or thereabout, of salvarsan died 1 to 2 days sooner than the controls, which died in 6 to 7 days. This suggests a possible earlier injury of the kidneys by the drugs, giving the leptospiras an easier and earlier access to, and localization in this organ. The inefficacy or dubious therapeutic value of salvarsan and neosalvarsan against the experimental *icteroides* infection of guinea pigs presents a close analogy to the observations already made by several investigators with *Leptospira icterohæmorrhagiæ*.

Several series of test-tube experiments were also made to determine the direct effect of salvarsan and neosalvarsan on *Leptospira icteroides* cultures. It was found, the injurious effect of alkalinity being eliminated, that the leptospiras remain motile for at least 1 hour in a concentration weaker than 1:10,000 of salvarsan or 1:1,000 of neosalvarsan. But they become gradually sluggish and succumb to the effect of the drugs at the end of 18 to 24 hours. The highest dilution which killed the leptospira in 18 hours was somewhere near 1:200,000.

When added to a culture medium, salvarsan and neosalvarsan both suppressed the growth of *icteroides* when their concentration in the

medium was 1:200,000. Hence these two drugs are highly poisonous for *Leptospira icteroides*.

The serums derived from rabbits which received 0.05 gm. of salvarsan or neosalvarsan per kilo of body weight 1 hour before bleeding proved to be very different from a normal rabbit serum in their behavior toward *Leptospira icteroides*. In the salvarsanized or neosalvarsanized serums the leptospiras remained active for at least 1 hour but appeared somewhat sluggish at the end of 18 hours, and were all dead and degenerated when examined after 48 hours. On the other hand, the leptospiras mixed with normal rabbit serum lived well and multiplied during the same period of time and under otherwise identical conditions (at 28°C.) To these tubes another portion of culture was added to determine whether or not a rapidly detrimental toxic substance had appeared in the drugged serum while standing for 72 hours, but the organisms remained still active at the end of 1 hour, 24 hours being required to kill them. In another experiment the salvarsanized and neosalvarsanized serums, together with normal serum as a control, were first left standing for 72 hours, after which period a rich culture of *icteroides* was introduced. The organisms remained uninfluenced for 1 hour in all the serums, but at the end of 24 hours many of those in the drugged serums were dead, and none was left alive at the end of 48 hours. In normal serum they steadily increased in numbers and were all active.

It is evident, then, that salvarsan or neosalvarsan introduced intravenously into the body of the rabbit is present in some form in the blood serum drawn at the end of 1 hour. The substance present in such serum has a slowly operating injurious effect upon *Leptospira icteroides*. The action of the drugs seems to be slower after passage through the animal body than before. If this phenomenon were to take place also in the infected body injected with these drugs, it is obvious that in a rapidly evolving infectious disease like yellow fever the progress of the infection will be too rapid to allow the drugs to exert their beneficial effect upon the course of the disease.

In direct contrast to the behavior of salvarsan and neosalvarsan *in vivo* and *in vitro*, anti-*icteroides* immune horse serum in a dose of 0.0001 cc., or 1 cc. of a 1:10,000 dilution, protected guinea pigs from an infection with at least 5,000 minimum lethal doses of *icteroides*

when injected simultaneously, but the same serum failed to exert any injurious effect upon the organism when mixed *in vitro* in a concentration weaker than 1:2,000. A rapid disintegration resulted with a concentration of 1:20 and almost complete agglutination and degeneration in 1:200.

The contrast between chemotherapy, as carried out with salvarsan and neosalvarsan, and serotherapy demonstrated with an immune serum is apparently of considerable practical significance.

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EXPERIMENTAL SYPHILIS IN THE RABBIT.

IV. Cutaneous Syphilis.

Part 1. Affections of the Skin and Appendages.

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Plates 47 to 58.

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In the preceding papers of this series (1–4), experimental syphilis in the rabbit has been presented from the standpoint of the phenomena of the infection which develops about the site of the inoculation. Ordinarily, these are the only manifestations of infection which are observed in the rabbit, but under certain circumstances, a generalized disease may be produced which is analogous in many respects to that of man, and in taking up the generalized infection, it will be necessary to give a brief résumé of the development of this phase of the subject in order that our work may appear in the proper relation to that of other investigators.

When it had been clearly established that the syphilitic infection could be transmitted to rabbits and maintained with undiminished virulence through successive generations of transfers, interest began to be centered upon problems of a generalized infection. From the outset, opinion was divided as to whether the infection in the rabbit became generalized as in man or remained essentially a localized infection. This division of opinion was in part an outgrowth of the controversy in regard to generalized infections in the lower monkeys and in part a result of experimental observation.

At all events, the development of lesions at points remote from the site of inoculation appeared to be an exceptional occurrence. The first instance of an infection of this kind was reported by Grouven in a series of communications beginning in 1907 (5–8). This case has become classic, and it is worthy of note that it still is the most pronounced example of a generalized infection following local inoculation which has been recorded in the literature. In fact, with a few exceptions, this animal exhibited all the manifestations which have since been

recognized as characteristic of generalized syphilis in the rabbit. In brief, the conditions noted included weakness, emaciation, dyspnea, alopecia, papular and maculopapular lesions of the skin, infiltrations and rhagades about the anterior nares associated with a mucopurulent discharge, conjunctivitis, a metastatic keratitis, and an infection of the testicles, epididymis, and regional lymph nodes, all developing from a unilateral infection of the eye and subsequent enucleation of the infected organ. The first manifestations of a generalized infection appeared 8 months after inoculation and active lesions were still present when the animal died 9 months later.

These observations of Grouven stimulated a great deal of discussion concerning generalized syphilis in the rabbit. Grouven himself reported two other cases (8), and in 1909, a fourth case consisting of a double keratitis originating from a testicular inoculation was reported by Menzincescu (9). About the same time, Uhlenhuth and Mulzer (10) reported a case of metastatic involvement of the scrotum of an uninoculated testicle with papular lesions about the anus, and Truffi (11) reported two cases of keratitis in animals inoculated in the scrotum. Subsequently, other cases of generalized infection were added to this list, but the total number of cases recorded in the literature has remained comparatively small, and most of the animals have shown only a few lesions of a minor character.

In addition to occasional instances of animals showing generalized lesions, there were other facts, however, which indicated that the infecting organism itself was not confined to the region of inoculation. As has been mentioned, Ossola (12) and Truffi (11) demonstrated the presence of spirochetes in the inguinal lymph nodes of rabbits inoculated in the scrotum in 1909, and as early as 1908, Neisser (13) reported successful inoculations of monkeys with material from the spleen and bone marrow of three out of seven rabbits killed 7 to 8 weeks after inoculation with a spleen-bone marrow emulsion from monkeys. These rabbits had been inoculated in the testicles, and although no local lesions had been detected up to the time they were killed, it was claimed that the virus was recovered from the internal organs. As reported by Neisser, these experiments might not be regarded as conclusive, since the only evidence of a specific infection submitted was the production of lesions in the inoculated monkeys which were said by Siebert to be typical primary lesions. At first, other investigators had difficulty in verifying these results, but eventually Truffi (14) succeeded in one instance (number of attempts not stated) in producing a definite infection by inoculation of bone marrow from an animal infected in the scrotum, and similar results have since been obtained by other investigators, notably by Uhlenhuth and Mulzer (15). The obvious objection which might be raised to these experiments, however, is that the results obtained appear to have been inconstant, and no one could say what proportion of the animals would have developed generalized lesions had they been permitted to live.

The occasional case of an animal with manifestations of a generalized infection or the recovery of the virus from the blood and internal organs of even a small

proportion of infected animals was sufficient, however, to establish the fact that generalization of the virus did occur in some instances, but no large series of experiments was carried out, so far as we are aware, for the purpose of determining the time and frequency or the extent of the generalization which took place, and this type of information was much needed to place the question of a generalized infection upon a proper basis.

Further than this, there has been little effort to correlate the phenomena of generalization and the production of generalized lesions. All that was determined was that some animals did show a dissemination of the virus, but in the experience of most observers the appearance of lesions other than those at the site of inoculation was the exception rather than the rule. For example, out of some hundreds of rabbits studied by Uhlenhuth and Mulzer (15), there were comparatively few which showed generalized lesions other than a metastatic infection from one testicle to the other or an occasional case of keratitis, and again Baeslack in 1916 (16) reported that there were no cases of generalized lesions in about 800 rabbits which he had studied.

On the other hand, a few investigators have observed generalized infections with greater frequency. In 1914 Nichols (17) reported the occurrence of generalized lesions of various types in one-half of a series of animals inoculated with a nervous strain of *Treponema pallidum*. Among the conditions mentioned were papular lesions of the eyelids, metastatic lesions of the scrotum and testicles, and lesions of the eyes. In this last group, there was included a new form of lesion of the eye ground recognizable by ophthalmoscopic examination. Unfortunately, neither the relative nor the absolute frequency of the various types of lesions was given, but the investigations were continued by Reasoner (18) who mentions among the manifestations produced by this organism "fundus involvement in about 75 per cent of the rabbits, extension to the opposite testicle in 10 per cent of cases, keratitis occasionally, involvement of the nasal mucosa frequently, with the presence of organism in the nasal discharge. Periostitis of the nasal bones is a later manifestation. There are eyelid lesions in 5 per cent. This strain also may cause mucous lesions of the penis and sheath. It occasionally causes a paronychia, and often a moderate degree of alopecia." But here again, Reasoner frequently refers to the use of repeated and combined testicular and intravenous inoculations, and it is not clear whether the facts recorded above represented results of testicular inoculation alone or included also results obtained from mixed and repeated inoculations of various kinds. In his conclusions, he states that "ordinarily the rabbit develops only an initial lesion, following inoculation in the genitalia, eyes and eyebrows."

On the whole, therefore, our knowledge of generalized syphilis in the rabbit resulting from local inoculation has rested to a large extent upon a proven dissemination of the infecting organisms, the time, frequency, and extent of which were unknown, and the occasional development of a few lesions at some point more or less removed from the site of inoculation, but no comprehensive description has been given of the infection thus produced.

Uhlenhuth and Mulzer (19, 20) described a generalized infection in the rabbit produced by a distinctly different mode of procedure, but even here the picture presented is incomplete. Since these investigators were unable to obtain a severe generalized infection from a local inoculation, they attempted to produce such a condition by resorting to a generalized inoculation, using intracardial and intravenous injections of very large doses of organisms. In this way, they succeeded in producing with considerable frequency what were in reality multiple primary lesions, first in young rabbits and then in adult animals. Considering the mode of inoculation employed, these infections were, of course, more analogous to intrauterine than to postnatal infections. Clinically, they were characterized by emaciation and weakness, especially in young rabbits, associated with lesions of the skin and appendages, the mucous membranes, the genitalia, the eyes, and tumor-like masses about the nose and tail. The extent of the involvement as well as the number and character of the lesions present varied considerably in different animals; there was an especial tendency to exudative affections of the mucous membranes, and the nose and tail tumors together with affections of the nails were the chief additions to the list of syphilitic manifestations which had been previously described. In other words, the lesions produced in these animals were of much the same character as those which had been reported in cases resulting from local inoculation but were, on the whole, more marked and could be produced with much greater frequency.

These experiments appear to have been accepted as a basis for constructing the picture of generalized syphilis in the rabbit and lent considerable support to the contention that marked generalized infection from local inoculation did not occur except in an occasional animal. Of course, the fallacy in this line of reasoning lay in the apparent assumption that a widely disseminated primary infection such as that produced by Uhlenhuth and Mulzer was comparable to conditions which obtained in the secondary diffusion of organisms from an established focus of infection. This error of conception, as will be seen, was a very serious one and had the effect of diverting attention from fundamental problems of the infection.

Since the publication of the work of Uhlenhuth and Mulzer, interest in experimental syphilis has abated somewhat and the only contributions of note which have appeared during the past few years are those of Nichols and of Reasoner.

While these earlier observations were doubtless correct as far as they went, the impressions created were founded upon an imperfect understanding of the experimental infection; they not only left unsettled many questions relative to the generalized infection but gave an erroneous impression as to the nature of the infection produced by local inoculation.

We have been able to collect a large amount of material bearing upon this phase of experimental syphilis which will be presented in

the following series of papers dealing with clinical manifestations of generalized syphilis in the rabbit. We regret that the pathology of these conditions cannot be presented in connection with their clinical history. We have, however, an abundance of material and hope that eventually we may be able to return to this phase of the subject and supply the pathological connection.

Source and Nature of the Material Studied.

The material which forms the basis for our description of generalized syphilis in the rabbit is composed of two groups of cases. Up to September 1, 1919, we had been able to collect 126 rabbits with outspoken manifestations of generalized syphilis developing from local inoculations in the testicles or scrotum. This list of animals included only those in which visible or palpable lesions were present at points entirely removed from the site of inoculation. Thus, metastatic lesions of the testicles and scrotum were not included among the generalized affections on account of the possible confusion of true disseminated lesions with extensions or transformations of processes arising from the primary focus of infection.

This first series of animals came from stock transfers and routine experiments connected with the use of the experimental infection in the rabbit as a means of studying the action of drugs against syphilitic infections and was composed of three classes of animals: (1) those in which the infection ran an undisturbed course; (2) those in which the generalized lesions developed subsequent to castration or excision of primary lesions; and (3) those in which the lesions followed drug administration.

From observations made in the course of routine work, together with an analysis of this group of cases, it appeared that the entire problem of animal resistance to *pallidum* infections, and hence the occurrence of generalized lesions, was intimately connected with the nature and extent of the reaction which took place at the site of inoculation, and that any influence which was capable of modifying this reaction might be expected to react upon the phenomena of the infection as a whole. In particular, it appeared that any condition which tended to lessen, to restrain, to inhibit, or to suppress this reaction without exerting a comparable effect upon the organisms them-

selves might be expected to favor the production of generalized manifestations of disease.

Accordingly, a series of experiments was carried out, aimed primarily at the fundamental problem of the nature of the animal infection and the mechanism of animal resistance. Thus, it was found that such simple means as a unilateral instead of a bilateral inoculation, unilateral or bilateral castration, or the use of therapeutic agents capable of inducing resolution of the primary lesions without destroying the infecting organisms, would completely alter the character of the infection and lead to the production of generalized lesions in a very large proportion of the animals inoculated.

From these experiments, a second group of animals with generalized syphilis has been collected which places the total number of cases available for study at well over 200.

The study of these animals was chiefly clinical. In the majority of cases, the lesions were allowed to pursue an uninterrupted course; in individual instances, they were excised for histological study, and some animals were killed at various periods of the infection in order to make a more thorough examination of existing conditions. The period of observation varied very considerably. Under the circumstances of our work, it was impracticable to hold most of these rabbits for any great period of time. The length of observation in most instances varied from a few weeks to several months; some animals were held for a year to 18 months and a few for more than 2 years.

The identification of syphilitic lesions rested upon the general character and clinical course of the lesions, the demonstration of spirochetes, and histological examination. In a few instances, therapeutic tests were used in the study of conditions of doubtful or uncertain character and as confirmatory diagnostic measures.

The manifestations of disease which we were able to recognize during the life of the animal fell into the five following groups and will be considered in this order: (1) affections of the skin and appendages; (2) affections of the mucous membranes and mucocutaneous borders; (3) affections of tendons, tendon sheaths, periosteum, cartilage, and bone; (4) affections of the eyes; and (5) lymphadenitis. In addition, certain visceral lesions, notably of the heart and of the central nervous system, were discovered at autopsy. This group of conditions will be reported later.

In presenting this material, we shall confine ourselves for the present to a general presentation of the subject, since it has been found that the incidence of different groups of affections, the character of the lesions present in a given case, their time of occurrence, and the general course of the disease are all more or less influenced by the circumstances under which the generalized disease makes its appearance.

Lesions of the Skin and Appendages.

The first group of conditions to be considered are the affections of the skin and appendages, including alopecia, paronychia, onychia, and lesions of the skin proper. Affections of this class occurred in a large proportion of the animals showing manifestations of generalized syphilis, and it will be necessary to divide the subject matter of these conditions into two papers the first of which will be confined to a description of the individual affections, while the second will deal with the clinical aspects of cutaneous syphilis.

Alopecia.

Local or general roughening of the coat and falling out of the hair are among the conditions most frequently mentioned as manifestations of generalized syphilis in the rabbit and are extremely common among rabbits infected with *Treponema pallidum*, but occur also from many causes in uninfected animals, the chief ones being moulting, diseases of the skin, systemic disease, and uncleanly habits. While we are reasonably certain, therefore, that a large number of our rabbits showed abnormalities of the coat referable to their syphilitic infection, we realize that it is very difficult to identify such cases with absolute certainty. In studying these affections, we first attempted to exclude the four causes mentioned, which can be done without much difficulty in all except the moulting, or shedding, of the animal. It was found that under laboratory conditions, rabbits were very irregular in this respect and might shed their coats at almost any time from early March to late November, which left only about 3 months of the year during which one was not constantly confronted by this possibility. As far as we were able to determine, there was no pathognomonic sign of syphilitic alopecia in the rabbit. Among the animals studied, there were comparatively few in which we felt justified in making such a diagnosis, and in nearly all instances the diagnosis was supported by the presence of other lesions whose character could be established with absolute certainty.

As nearly as could be determined, there appeared to be at least three conditions which might be regarded as syphilitic alopecia. The most common of these

was one in which the coat became roughened or unkempt; the hair was dry and without luster and was continually falling out. This condition might affect the entire coat, as in Fig. 1, or only some smaller area, and was especially common about the head and ears, giving to these parts a characteristic moth-eaten appearance (Fig. 2). This form of affection is probably the one usually referred to as syphilitic alopecia in the rabbit.

No true baldness was noted in affections of this kind. In exceptional instances of diffuse alopecia, there was decided thinning of the hair over certain areas of the body such as the thighs, the thorax, the abdomen, or about the elbows (Fig. 3). Facial alopecia, alopecia of the ears, and of the cheeks were more marked as a rule, and the hair frequently came away from these areas in considerable masses (Figs. 3 and 4) or with gentle rubbing patches were left which were entirely denuded of hair.

A second form of alopecia which might be referred to a syphilitic infection was characterized by no other manifestation than looseness of the hair. The coats of these animals appeared to be in perfect condition but upon gently plucking at the hair, it was found to be so loose as to come away in handfuls, leaving the skin perfectly bare or covered with a short stubby growth of hair as in Figs. 5 and 6. This condition was so marked in some animals that it was possible to pluck the fur from large areas of the body without inflicting the slightest traumatism to the skin. In some instances, the skin itself appeared entirely normal, while in others, removal of the hair revealed the presence of unsuspected lesions (Fig. 5). A similar condition was found to occur in some animals at the time of shedding. In these cases, however, the growth of new hair was nearly always well advanced before the old hair could be removed, which was not the case with the supposedly syphilitic affection.

Both these forms of alopecia were only temporary affections as a rule, and after a few weeks or months, the coat returned to a normal condition. They appeared to be intensified, however, at the shedding periods of normal rabbits, which in itself is not surprising. In one animal of our series, which was held under observation for about 18 months, there was no time at which the entire region over the hips, thighs, and loins could not be picked entirely free of hair, and as fast as the hair returned, it could be plucked out again with but the slightest tension. A similar condition existed over other portions of the body, but otherwise the coat appeared to be in remarkably good condition, as may be seen by reference to Fig. 6.

A third form of alopecia which bordered upon a true baldness was seen in a few animals. This condition was usually confined to an area a few centimeters in diameter and was characterized by what might be called a peeling of the fur, the roots of which were matted together by superficial layers of the epidermis. By rubbing these areas, masses of fur came away, leaving a bare skin covered with fine epithelial scales. The skin showed a variable degree of thickening, and in some instances focalized infiltrations and necrosis as well. A typical

though rather pronounced case of this kind is illustrated in Fig. 8. The condition here shown extended from the cheeks down over the neck and shoulders where there were two well defined areas of infiltration and necrosis of the skin.

With recovery of these lesions, there was a thin stubby growth of hair but no return to normal as in the preceding cases. This condition might be classed as a true skin lesion rather than as an alopecia, but the alteration in the skin was so variable and in some instances so slight that the alopecia appeared to be the most characteristic feature of the condition.

There was one other condition noted which may be mentioned, the etiology of which could not be determined with certainty. It affected the hair of the face and especially that over the bridge of the nose, and was characterized by a thinning of the hair over the affected area while that remaining appeared broken and irregular much as in the case of tinea infections (Fig. 7). The skin of the affected part showed a slight infiltration in some cases, but in others was thin or atrophic. The only causative factor which suggested itself besides that of a specific infection was the rubbing of the animal against the cage, and this seemed unlikely. Once the condition developed, there was little or no tendency toward a return to normal.

Onychia and Paronychia.

Onychia and paronychia have been mentioned among the manifestations of generalized syphilis in the rabbit, but in our experience, affections of the nails which could be definitely ascribed to syphilitic infection were comparatively rare—only seven cases having been recorded among 126 rabbits and only one of these could be regarded as a true onychia.

Paronychia was first recognized by a slight reddening and swelling of the skin about the base of the nails. The skin then became thickened or infiltrated and was covered with yellow or yellowish gray scales or crusts producing a condition like that shown in Figs. 9, 10, and 12. In extreme cases, the reaction about the base of the nail was much more marked and resulted in the formation of granulomatous masses which underwent secondary necrosis and ulceration with consequent disturbance of the nutrition of the nails themselves (Figs. 11 and 12).

These conditions were usually bilateral and symmetrical and affected the nails of both the front and hind feet.

Syphilitic paronychia was found to be difficult to distinguish in some instances from a non-syphilitic affection of a similar character which is comparatively common among rabbits. This fungus or parasitic disease of the nails develops sooner or later in all rabbits with fungus or parasitic infections of the skin or external ear. While

these affections occur upon the hind feet as well as the front, they usually make their appearance about the toes on the median side of the front feet and are not confined to the region of the nails but spread diffusely over the interdigital surfaces. In contrast to this, syphilitic paronychia appeared to be more common about the nails of the lateral toes and was sharply confined to the base of the nail. Again, the parasitic disease is a steadily progressive affection and never clears up spontaneously, while the syphilitic condition is variable in its course and clears up completely without any treatment. If spirochetes can be found, they are helpful in making a diagnosis, but in their absence, one has to rely upon clinical characteristics.

A true onychia, as has been mentioned, was recognized by naked eye observation in only one of our first group of rabbits. In this case, the condition was associated with a marked paronychia of some of the toes, and the alterations in the nails were so pronounced as to leave no doubt as to the etiological factor concerned (Fig. 11). A similar but less marked condition was also presented by the animal shown in Fig. 12. In other instances it was noted that the nails showed signs of wearing short or appeared roughened and broken towards their ends and tended to split and scale just as in the case of the outer and inner toes of the animal in Fig. 11. An example of nail involvement of this type with no associated paronchyia is shown in Fig. 13.

At the time these observations were made, no relation could be established between this condition and the syphilitic infection. Subsequently, however, it was learned that alterations such as these might arise from involvement of the nail bed. In the rabbit, the nail fits closely over the terminal phalanx, and periostitis with considerable destruction of these bones may take place without giving rise to any external evidence of the existence of such an infection other than an alteration of the nails of the affected toes.[1] Onychia in the rabbit appears, therefore, to be most often associated with a periostitis and is difficult of recognition by the use of ordinary means of diagnosis. If the involvement is slight, the nail may eventually recover, but if the destruction is extensive, the effect appears to be permanent or at least of long duration.

[1] These observations were made by Dr. W. H. Brown, Dr. L. Pearce, and Dr. W. D. Witherbee in the course of a series of investigations of deep seated bony changes by the use of the x-ray, the results of which will be reported later.

Cutaneous Lesions.

The cutaneous lesions formed a very large and varied group of affections. They included lesions of the macular, papular, and nodular or tubercular varieties, and while they possessed many features in common with the cutaneous lesions of man, they differed from them in so many respects that it would be difficult to attempt a complete correlation of the two classes of lesions upon the basis of the material which is at present available. The only classification of cutaneous lesions of the rabbit which seems justifiable at this time is one based upon very broad lines of differentiation such as that afforded by developmental or pathological characteristics.

As was pointed out in connection with scrotal infections, one of the most striking features of the skin reaction of the rabbit to localized infections of *Treponema pallidum* is the tendency to proliferation on the part of the fixed tissue cells and the formation of large granulomatous lesions; a second characteristic of the skin reaction is the tendency to a more or less diffuse infiltration associated with varying degrees of desquamation of surface epithelium, exfoliation, and surface erosion, or necrosis and ulceration; while a third feature of the reaction is a localized hyperemia or even hemorrhage which is associated with varying degrees of exudation expressed chiefly in the form of an edema.

These characteristics of the primary reaction in the scrotum find their counterpart in the reaction to localized infection in other skin areas and form, therefore, an acceptable basis for a consideration of the cutaneous lesions of generalized syphilis. Upon this basis, cutaneous lesions will fall into three classes, the hyperemias, the infiltrations, and the granulomata. It should be understood, however, that there is no sharp line of distinction between these three classes of conditions as there is always a tendency to a combination of the three processes, and such distinctions as can be made must be based upon the predominance of one or another of the three forms of reaction.

In taking up the discussion of cutaneous lesions, the above sequence will be reversed in order to begin with those conditions which are the most obvious; namely, the granulomata.

Cutaneous Granulomata.

The lesions classed as cutaneous granulomata (Figs. 14 to 20 and 22) included those affections which developed in consequence of a reaction which was marked by proliferation of fixed tissue cells and the formation of circumscribed elevated nodules of a fleshy character varying from a few millimeters to several centimeters in diameter. Lesions of this type were quite common. They usually occurred singly or in small groups and as a rule were few in number, but occasionally they were fairly numerous and as many as twenty-eight lesions have been counted in a single animal at one time.

The granulomata appeared either in the form of rather diffuse areas of thickening which involved a considerable depth of the skin or as sharply circumscribed and indurated nodules (Figs. 14 and 17). The more diffuse lesions and the larger nodules were usually of a rose-pink or copper color or were paler than normal and of a faint yellow color, while the small discrete nodules were generally pale and opaque or of a decided opalescent appearance. In exceptional instances, the early lesion was of a deep violet-red color or appeared almost as thickened purpuric spots in the skin. In general, the surface of the lesion was smooth and rather translucent, but during the early stages of its growth there was no marked disturbance of the hair covering its surface. These several conditions are illustrated in the accompanying photographs (Figs. 14 to 19) which show various forms and developmental stages of typical granulomatous lesions.

The evolution of the cutaneous granulomata was usually rapid and within a week or so led to the formation of large oval or irregular spherical masses showing various types of secondary alteration such as are illustrated in Figs. 14 to 23. It is important to note, however, that not all the lesions present in a given case exhibited these changes to an equal degree. As a rule, only a few of those present developed to any considerable extent, while the others underwent involution and completely disappeared. This is a phenomenon worthy of note, since it illustrates the inhibitory influence exerted by one lesion or group of lesions upon another, as was pointed out in connection with the development of multiple scrotal lesions.

The growth of granulomatous lesions took place chiefly from the deeper layers of the skin and tended to be of a concentric type so that surface alterations were usually confined to a relatively small area. The most common changes observed were those due to necrosis. In some instances, this was very superficial and produced no more than a slight exfoliation of epithelium or surface erosion; in others, it extended to a greater depth, and the skin over the center, or even a larger portion of the lesion, became converted into a dry, adherent crust, or a depressed ulcer was formed which converted the lesion into a typical chancre-like mass.

While, as a rule, the area thus involved was small, occasionally a large part of the lesion was destroyed in this manner—the necrosis keeping pace with the growth of the lesion (Figs. 18 and 19).

Another noticeable feature of this class of affections was the preservation of the hair over a large part of the lesion which was obviously due to the nature of the skin involvement. With the advent of infiltration and necrosis of the outer layers of the skin, the hair first became thin and broken and was then lost over the affected area, but, as may be seen by reference to Fig. 19, many lesions showed practically no disturbance of the hair outside the zone of necrosis. In other instances, however, there were a more extensive involvement of the outer layers of skin and an obliteration of papillæ extending well beyond the area of necrosis. In these cases, the loss of hair was more pronounced and lesions were formed which, with their smooth and infiltrated surfaces, bore an even more striking resemblance to the typical scrotal chancre (Fig. 20). Usually the obliteration of skin papillæ and the loss of hair merely preceded the appearance of other changes, but not infrequently they marked the extent of the surface alteration, and the lesions thus formed were quite analogous to unulcerated nodular chancres of the scrotum (Fig. 22).

In many ways, the cutaneous granuloma of the rabbit may be viewed as an expression of a vigorous reaction to infection analogous in all respects to that which characterizes the reaction at the primary focus of infection. Objectively, the force of this statement may be appreciated by comparing Figs. 20 and 21 and Figs. 22 and 23 which represent corresponding cutaneous and scrotal lesions of two animals photographed at the same time. The significance of the striking similarity between these two processes of reaction will be made clear when we come to consider the factors in animal resistance and the mode of expression of this resistance.

Before leaving the subject of cutaneous granulomata, reference may be made to a similar group of lesions which originated within the subcutaneous tissues and reached a considerable size before any skin involvement took place (Fig. 24). These lesions were, of course, more analogous to ordinary gummata, or the so called tertiary skin lesions of man, than to those of earlier stages of the disease, and the few cases seen in the rabbit also appeared late in the course of the infection. For example, the animal shown in Fig. 24 had a succession of cutaneous lesions, the first appearing about $3\frac{1}{2}$ months after inoculation. There were no lesions in the subcutaneous tissues, however, until 25 months after inoculation when the lesion upon the nose developed and was followed 3 months later by other lesions of a similar character.

In all granulomatous lesions, spirochetes were numerous and could be demonstrated without difficulty as long as the lesions were active.

Cutaneous Infiltrations.

The second group of cutaneous lesions to be described (Figs. 25 to 41) includes a variety of conditions ranging from small discrete papules on the one hand to large weeping or crustaceous patches on the other, the common basis of which was a cutaneous infiltration in contradistinction to the proliferative reaction which characterized the lesions of the preceding group. According to available data, these affections occurred among our first series of animals about as frequently as the granulomata, but their incidence has steadily declined with changing conditions until, under present circumstances, they might be regarded as comparatively rare affections.[2] It should be pointed out, however, that lesions of this class were often less clearly defined and much more difficult of detection than the granulomata, and hence it is not unlikely that many may be entirely overlooked.

At the time of their appearance cutaneous infiltrations may differ very slightly if at all from early granulomatous lesions. As in the case of the granulomata, two forms of lesions could be distinguished from their mode of origin, one appearing as a somewhat diffuse process and the other as a very minute and sharply circumscribed nodule, and these characteristics tended to be preserved in the fully developed lesions of this class. There were thus formed two fairly well defined groups of conditions, the one a flattened or lenticular lesion, the other a more elevated and indurated papule.

Flattened Papular Lesions.—The initial lesion of this group (Figs. 25 and 26) presented the appearance of a simple infiltration of the skin, involving the papillary layers and varying from a few millimeters to a centimeter or more in diameter. These spots were usually of a faint pink or copper color, but in exceptional instances, the discoloration of the skin was quite pronounced, approaching in intensity the violet-red color occasionally seen in early granulomatous lesions (Fig. 26). At this stage, the affected area was raised but slightly above the surrounding skin level, the elevation being greatest at the center of the lesion and diminishing towards its periphery.

[2] Changing conditions in the life history of the organisms and the particular circumstances under which generalized lesions make their appearance in a given case are undoubtedly potent factors in determining the character which these lesions assume.

Very soon the appearance of the lesions changed, the color becoming a pale yellow or gray or deepening to a copper or brown as the case might be. At the same time, the infiltration increased and the hair over the affected area was lost to a considerable extent, while the skin became smooth and glistening and of a parchment-like consistency or was covered by thin scales of a yellow or yellowish gray color (Fig. 27).

Subsequently, numerous modifications of these lesions occurred as a result of growth or extension or from secondary alterations taking place within the affected area. The characteristic mode of growth of the flattened papular lesion was a peripheral extension of the infiltration which in itself gave rise to a variety of conditions. Most often there was produced a small but fairly well defined area of infiltration analogous to some of those shown in Figs. 28 and 31. Occasionally, however, the process assumed more extensive proportions and from one or more small lesions, there developed a widespread affection such as that seen in Fig. 28. Not infrequently, the original lesions or the center of the lesion in such cases underwent resolution with the peripheral extension of the process as may be seen by an examination of Figs. 28 and 29. There was thus formed a single annular lesion, as in Fig. 29, or an affection composed of a series of lesions grouped in similar fashion about the area in which the infection first appeared (Fig. 28).

In addition to conditions such as those described, numerous modifications of these lesions occurred which were attributable to secondary alterations. Among these may be mentioned a squamous type of lesion, a suggestion of which is given by the accumulation of scales about the margins of the lesion in Fig. 29. Another condition which was of frequent occurrence in this class of affections was exfoliation of the epithelial coverings which gave rise to moist or weeping patches or to areas of infiltration covered by thick yellowish gray or yellowish brown crusts (Figs. 31 and 33). In other instances, necrosis of a more pronounced character occurred and resulted in the formation of an ulcer, as in one of the lesions in Fig. 29, or the entire area of infiltration was converted into a dry, necrotic eschar, a condition which is suggested in Fig. 8.

While in many instances the flattened papular lesions were rather small and few in number, they were occasionally quite numerous and tended to spread and to fuse with one another, as has been described, or were processes of a rather diffuse character from the beginning and covered an area several square centimeters in extent. As might be expected from the nature of the process, this particular type of lesion was especially prone to exfoliation and necrosis and the formation of weeping patches or of lesions of a crustaceous character such as that shown in Fig. 33.

As was pointed out in connection with the cutaneous granulomata, it is important to note again that many of the infiltrative processes which have been described are closely analogous to certain forms of primary scrotal lesions. In order to emphasize this point, two sets of photographs have been inserted to enable one to make the comparison between the primary and the generalized

cutaneous reactions as they appeared in the same animal (compare Figs. 29 and 30, and 31 and 32).

Raised Papular Lesions.—The raised papular lesions of the skin (Figs. 34 to 41) analogous to the miliary or follicular syphilides of man were of three varieties. The simplest of these and the one from which the others usually developed was a small shotty nodule averaging from 1 to 3 mm. in diameter and raised a millimeter or so above the surrounding skin level (Figs. 34 to 36 and 38). These lesions were very sharply demarcated and were of a grayish white or faint copper color. In most instances, they were rather dense and opaque but occasionally were of a semitranslucent or opalescent appearance. The crest of the lesion tended to be rounded or pointed, but in exceptional instances there was a slight umbilication as in Fig. 36. Some of the lesions were also surrounded by a rich vascular network as is clearly shown in this same figure (Fig. 36).

These papules rarely persisted in the condition described for any considerable period of time but tended to undergo one of two types of alteration. Many of them underwent a central or apical necrosis with the formation of a small crust or ulcer. This was particularly the case with lesions situated about the face or ears (Fig. 37). The second modification seen was of a squamous type, and this was especially common with lesions of the upper eyelids and brows (Figs. 38 to 41). In some of these, a thick layer of epithelial scales was formed upon the crest of the lesion (Fig. 40), while in others, the entire nodule was incased in a covering of horny epithelium (Fig. 39). This group of conditions presented the general appearance of small vegetations, and their prominence was due largely to the accumulated epithelial coverings which upon removal left an unexpectedly small and rather flattened body of infiltration.

Another feature of these lesions was the tendency to occur in groups such as those shown in Fig. 39. In two animals of our series, one of which is shown in Fig. 37, the papules showed a definite circinate arrangement and a very striking bilateral symmetry.

Routine examinations for spirochetes were not made upon all the lesions of this group which were encountered, since it was desired to follow the clinical course of the lesions as far as possible, and it was found that the traumatism inflicted either by aspiration with a needle and syringe or by scarification was sufficient to disturb the development of the smaller lesions and predisposed to rapid regression or healing. However, spirochetes were demonstrated in lesions of all the types described. They were most easily demonstrated in the moist or weeping patches of infiltration and in the fleshy papules but were more difficult to demonstrate in the dry necrotic lesions and the small follicular papules. When once familiar with this group of lesions,

however, the clinical history was usually sufficient in itself to enable one to make a perfectly definite diagnosis.

While, in attempting to point out the salient features of various types of cutaneous eruptions, a distinction has been drawn between cutaneous infiltrations and cutaneous granulomata, it must be said that no complete division between these two classes of affections could be made. In reality, they tended to merge one with the other, and while the great majority of the lesions seen in the rabbit could be assigned to one or the other of these two groups, there were borderline conditions which would be very difficult to classify upon this basis.

As our investigations advanced, the difficulties encountered in this respect were increased due to a change in the character of the lesions produced by the two strains of organisms employed. This was especially noticeable in a decrease in the relative incidence of lesions which have been described as infiltrations and a corresponding increase in lesions which clinically might be regarded either as small granulomata or as unusually marked processes of infiltration. Mention is made of this fact on account of the bearing which it may have upon any interpretation which may be placed upon such conditions as manifestations of a reaction to infection.

Macular Erythema, or Roseola.

A macular erythema has never been described among the cutaneous lesions of the rabbit so far as we are aware, but in the course of our own observations, a pronounced rash of this type was seen upon the ears of a number of animals with manifestations of generalized infection. As yet, no conclusive proof of the syphilitic nature of these lesions has been obtained. Thus far, we have been unable to demonstrate spirochetes in the lesions, although we have not had an opportunity of making a thorough search for them in section. The evidence which we have rests upon clinical and histological examinations.

In its typical form, the macular erythema first appeared as slightly thickened, rose-colored spots or patches situated in the deeper layers of the skin or in the subcutaneous tissues of the outer third of the ear. The color of the rash deep-

ened to a dusky red or purple, then changed to a coppery hue, and faded, leaving a faint stain in the tissues. Occasionally, there were small petechial hemorrhages associated with the lesions and a central opaque white spot or ring which persisted after the general color of the rash had faded. A typical though rather pronounced case of macular erythema is shown in Fig. 42.

As a rule, the hair was loosened over the affected area or over a much greater area than appeared to be involved, and, in a few instances, the surface of the skin was also affected, becoming roughened and covered with scales, a condition which was especially noticeable upon the inner surface of the ears where the skin is normally quite smooth. Ordinarily, no other changes were noted, but in two instances, there was a well marked infiltration of the affected areas which made its appearance just as the rash began to fade. The ear of one of these animals and the areas of infiltration are shown in Fig. 43.

In their distribution, the macular lesions usually preserved a fair degree of bilateral symmetry. Aside from the ears, the only positions in which definite purpuric spots of the character described have been seen were the trunk and the thighs of two animals.

During the early stages of the erythema, the color was diminished by pressure or by vascular constriction and increased with dilatation of the vessels, but such influence had little if any effect upon the appearance of the rash after the purpuric stage had been reached. In general, these lesions suggested a reaction similar to the early reaction described in the scrotum following inoculation with a virus emulsion.

This macular erythema was usually fleeting and rarely persisted for more than a few days, and frequently a slight roseola disappeared within 24 to 48 hours after it was first noted. In most instances, the roseola developed early in the course of the infection, but in one animal, it was observed as late as 16 months after inoculation. Another characteristic feature of these lesions was a marked tendency to recurrent periods of eruption.

After a long series of observations, it was found that a very large proportion of the animals which showed eruptions of this type belonged to one of three classes: first, those which had shown other manifestations of generalized infection, second, animals in which such lesions were still present, or third, animals which subsequently did develop lesions of a definite syphilitic character.

Histologically, three types of change were found in these lesions. In the early stages of the erythema, the vessels of the region were

dilated and the surrounding tissues were edematous. At a later period, there was stasis as well as dilatation of the vessels and slight extravasation of red blood cells along with the edema, and the endothelial cells of the vessels were swollen. These were the usual changes, but in the most marked cases, there were in addition a slight migration of leucocytes and an accumulation of round cells. These changes are precisely those which occur with the initiation of the specific reaction in the testicle and scrotum.

The chief source of difficulty in relating the macular erythema to infection with *Treponema pallidum* is the occurrence of erythemata and vasomotor disturbances of various sorts in the ears of normal rabbits. The non-specific condition usually develops from the base of the ears and radiates outward along the marginal vessels but may assume a macular form not unlike that of the early roseola described. These conditions were found to occur especially during periods of moulting, or shedding, and at such times, thickened erythematous patches may be found on any part of the body where new hair is beginning to grow. However, the purpuric type of eruption was never observed except in infected animals, although very many normal rabbits were examined with this point in view.

As the matter stands, therefore, we feel reasonably certain that a true macular lesion does occur in the rabbit, but the difficulties of making a positive diagnosis are so great that we have confined our listing of such conditions to animals which showed some other manifestation of generalized syphilis, and among these, to those animals in which the rash appeared in typical form.[3]

Lesions of Uncertain Etiology.

The cutaneous lesions thus far described include only those affections whose etiology we have been able to establish with absolute certainty or, as in the case of the macular erythema, with a strong degree of probability. This group of manifestations would hardly be complete, however, without mention of several conditions which may

[3] It is possible that therapy exercised some influence upon the occurrence of macular erythemata, since eight of the first thirteen animals in which an outspoken eruption of this character was observed, were drug-treated animals.

bear some relation to *pallidum* infections but whose etiology we have not been able to establish to our own satisfaction.

The first of these conditions is that illustrated by Fig. 44 which shows a bare area of skin in the lumbar region from which the hair has been plucked. In this area, there were two definite nodular masses, one covered by a short growth of hair, the other entirely bare and of a decided red color. A similar reddened and thickened area projects beyond the margins of the upper edge of the denuded skin area. These conditions were comparatively common and especially so at the time of moulting.

It will be noted that the peculiar condition of the skin in this animal is not unlike the early stage of a syphilitic skin lesion but analogous conditions are known to occur among uninfected rabbits. In the presence of a negative examination for spirochetes, obviously no diagnosis of the nature of such affections could be made. Granting, however, that conditions of this kind are entirely phenomena of a new growth of hair, it still appears possible that they may be influenced to some extent by the presence of a syphilitic infection. Whether such is the case cannot be said. The conditions were confined almost entirely to the face, where known skin lesions usually present a very characteristic appearance, and to the trunk, a region in which almost nothing is known of syphilitic lesions. The condition is mentioned mainly to avoid confusion between affections of proven origin and conditions of unknown etiology which may simulate them.

The second group of conditions to which we may refer concerns more especially certain of the hair follicles or possibly the sebaceous glands of the cheeks and, to a lesser extent, the neck. In a considerable number of infected rabbits, it was found that an abnormal condition of the skin developed about the roots of certain hairs, usually late in the course of the disease. The condition was characterized clinically by the formation of an exudate which glued together small tufts of hair irregularly distributed over the cheeks and sides of the neck. This material extended a millimeter or so up the shaft of the hair and resulted in the formation of dry, hard projections which were very easily palpable and were of a cream-yellow or lemon-yellow color. When the hair was clipped, the appearance presented was that shown in Fig. 45.

This condition might persist for a very long time or might clear up after only a short duration. In the process of clearing, the hair came out, and one found beneath these masses of exudate, faint circles in the skin similar to those seen in Fig. 46.

A condition which appeared to be of the same nature developed in several animals whose cheeks had been shaved as a means of investigating suspected skin lesions. One of these animals is shown in Fig. 47. The lesions formed under these conditions were minute points of elevation surmounted by a small crust. It was found that by applying pressure or tension to the skin, a small droplet of clear or slightly turbid fluid exuded from these points. This fluid contained a

large number of polynuclear and mononuclear cells, but no spirochetes could be found.

In this particular animal, it may be of interest to note the existence of another abnormality in regard to the growth of the hair of the area shown. This animal with two others was shaved several weeks before the photograph in Fig. 47 was taken. At that time, there were several small syphilitic lesions at the base of the ears. The hair of the other two animals (both with specific lesions) returned very promptly, but in this animal, there was practically no growth of hair for many weeks which in itself is indicative of disturbed nutrition of the hair, and is a condition which has been repeatedly observed in animals with marked generalized syphilis.

This group of conditions suggests the possibility of a follicular syphilide, possibly a pustular eruption, but at present, it is no more than a suggestion. Similar conditions were found in uninfected rabbits but were far less common and were usually associated with other evidences of abnormality. In infected animals, this condition showed no relation to other diseases. For example, the rabbit in Fig. 45 was in perfect condition except for the presence of two small lesions upon the hind feet. A year previous, however, it had been the subject of a most pronounced cutaneous syphilis.

We are of the opinion that some of these conditions may be directly or indirectly related to infection with *Treponema pallidum*. We have had no opportunity, however, to make a thorough investigation of them and in the absence of any definite proof of a syphilitic origin, they must be left as conditions of uncertain etiology.

SUMMARY.

From the study of a large series of rabbits with outspoken manifestations of generalized syphilis, lesions of the skin and appendages were found to constitute one of the largest and most varied groups of such affections. The conditions noted consisted of alopecias, onychia and paronychia, and lesions of the skin proper.

It was found to be a matter of some difficulty to make a positive diagnosis of syphilitic alopecia, but there were three and possibly four conditions which appeared to be attributable to such an infection. The first of these took the form of a general or local roughening of the coat with falling of the hair which produced the typical moth-eaten appearance associated with syphilitic alopecia in the human subject. A second form of alopecia was essentially an abnormal looseness of the hair which permitted large areas of the body to be completely

denuded. The third type of alopecia was associated with definite skin changes, and the hair was readily removable together with an adherent mass of epithelial scales.

Paronychia was comparatively rare but was readily recognized by a characteristic infiltration and exfoliation of the skin about the base of the nails.

The incidence of onychia is uncertain. Late in the course of the investigation it was found that alterations in the nails which were not entirely characteristic in themselves might occur in consequence of a syphilitic involvement of the nail beds which could not be detected by ordinary methods of examination. The cases which were recognized as syphilitic were those which showed an associated paronychia.

Lesions of the skin were found to be one of the most frequent manifestations of a generalized infection in the rabbit. These lesions were divided into three classes: first, granulomatous lesions, second, infiltrations, and third, erythemata.

The granulomata were lesions of a fleshy character which tended to grow to a very large size and presented all the characteristics of circumscribed primary lesions of the scrotum.

The conditions described as cutaneous infiltrations included two general types of lesions, one a flattened and rather diffuse process, the other an elevated and sharply circumscribed papule. As a class, these lesions were very prone to secondary alterations and in this way gave rise to a great variety of conditions which in general resembled the diffuse primary lesions of the scrotum and the papular lesions resulting from local dissemination.

A third type of lesion resembling the macular erythemata of man was observed in a small number of animals, and while no definite proof of the specific origin of these lesions was obtained, the evidence available was strongly suggestive.

In addition, several other cutaneous affections were noted which have not as yet been thoroughly investigated. It is suggested, however, that these processes may bear some relation to infection with *Treponema pallidum*.

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EXPLANATION OF PLATES.

All the illustrations are from unretouched photographs which, with the exception of Figs. 1 and 6, represent the objects at approximately their natural size.

FIGS. 1 to 8. Alopecias in the rabbit. Abnormalities of the coat which may be referable to a syphilitic infection. All the animals here shown had active manifestations of generalized syphilis, other than the alopecia, at the time these photographs were taken with the exception of one in Fig. 6 and in this case there was a double keratitis at the time the alopecia first appeared.

PLATE 47.

FIG. 1. An abnormal condition of the coat frequently observed in rabbits infected with *Treponema pallidum*. A diffuse alopecia.

PLATE 48.

FIG. 2. Alopecia areata, showing a slightly moth-eaten appearance of the hair about the face and ears. This condition may be simulated by ordinary processes of moulting.

FIG. 3. Diffuse alopecia, showing a marked thinning of the hair over the thigh and a slight roughening of the skin.

PLATE 49.

FIG. 4. Alopecia areata associated with desquamation of surface epithelium, a condition also seen at the time of moulting.

FIG. 5. An animal in which the hair was plucked from the region of the head and shoulders. Elsewhere the hair was firmly set. Note the cutaneous lesions on the face. An affection as pronounced as this is rarely observed in a normal animal but is comparatively common among those infected with *Treponema pallidum*.

PLATE 50.

FIG. 6. An affection of the coat characterized by abnormal looseness of the hair which persisted over a period of more than 18 months. The region over the hips, thighs, and loins of this animal were plucked clean a number of times. Note the excellent appearance of the coat of the animal.

PLATE 51.

FIG. 7. A peculiar thinning of the hair over the lower portion of the nose.

FIG. 8. The same animal as in Fig. 7. Alopecia areata associated with a slight diffuse infiltration of the skin, desquamation of epithelium, and focal necroses. An unquestionable syphilitic affection.

FIGS. 9 to 13. Affections of the nails.

FIGS. 9 and 10. A bilateral paronychia of the front feet.

FIG. 11. Left hind foot. Marked paronychia and onychia of the nails on the two middle toes and onychia of the inner toe. Hair clipped.

FIG. 12. Right hind foot. Paronychia of first and third toes. Onychia with loss of the nail. Hair clipped on the third toe.

FIG. 13. Simple onychia of hind foot. Hair over the toes clipped. This animal showed a periostitis and necrosis of the terminal phalanges.

PLATE 52.

FIGS. 14 to 19. Cutaneous granulomata analogous in many respects to the grouped nodular or tubercular lesions of the so called secondary and tertiary periods of human syphilis.

FIGS. 14 to 16. Stages in the development of a typical cutaneous granuloma as seen at intervals of 1 week. Fig. 14 shows an area of diffuse thickening in the skin, the surface of which was of a decided copper color. In Fig. 15, there is beginning surface necrosis, and Fig. 16 shows a well developed chancre-like lesion with central necrosis and depressed ulcer. Area shaved.

FIG. 17. A group of six early granulomatous lesions on the dorsum and side of the right hind foot with an older lesion over the tendo achillis as seen after removal of the hair. These lesions were all sharply demarcated but varied in appearance from pale opalescent nodules to nodules of a deep violet-red color. Note the tense appearance of the skin and glistening surface of the two largest lesions.

FIG. 18. A group of granulomatous lesions on the right hind foot representing various stages of development. The lesions in this animal were characterized by an intense violet-red color and by rapid and widespread necrosis. The enlargement of the fifth metatarsal seen in the photograph is due to a syphilitic periostitis. Hair removed.

FIG. 19. Granulomatous lesions of the foot illustrating various stages of necrosis and ulceration and the persistence of the hair over this class of lesion. Hair clipped.

PLATE 53.

FIGS. 20 and 21. A cutaneous granuloma and the scrotal lesions of the same animal. The animal was inoculated in the testicles and subsequently the left testicle was removed, but large scrotal lesions developed on both sides, presenting essentially the same appearance as the generalized lesions of the skin.

FIGS. 22 and 23. An unulcerated granuloma of 4 months duration with a bald patch on its surface and the corresponding lesion of the scrotum 11 months after inoculation. The two lesions were photographed at the same time. A similar lesion in the right scrotum had been excised.

FIG. 24. A subcutaneous granuloma, or gumma, freely movable between the skin and the nasal bones, $26\frac{1}{2}$ months after inoculation. Hair clipped.

FIGS. 25 to 41. Cutaneous infiltrations. The lesions in this group represent processes which are perhaps more analogous to the cutaneous lesions of man than those of the preceding group.

PLATE 54.

FIG. 25. Large copper-colored patches of infiltration just appearing on the right ear. Ears shaved.

FIG. 26. A very early but pronounced area of infiltration on the dorsum of the right front foot. The lesion was of a violet-red color and at this time presented much the same appearance as an early granulomatous lesion. The nodular mass at the carpus is a syphilitic lesion of the ulna. Hair removed.

FIG. 27. Small cutaneous infiltrations at the base of the ears showing accumulation of epithelial scales. An early secondary transformation. Area shaved.

FIG. 28. Multiple lesions of the fore arms and feet grouped in irregular circles with the most active lesions towards the periphery. The affection first appeared in the region of the carpus and subsequently extended as shown in the photograph. The lesions were profusely covered by fine epithelial scales most of which were unavoidably lost in shaving the affected area.

PLATE 55.

FIG. 29. The same animal as in Fig. 28. An annular lesion, the margins of which are partly covered with epithelial scales, and a smaller area of infiltration with a necrotic center covered by a scab. A third lesion is seen in profile upon the anterior surface of the ear. Area shaved.

FIG. 30. The primary lesions of the scrotum in the same animal as that in Figs. 28 and 29, intended to show a similarity in the cutaneous reaction of various parts of the body or between the so called primary and secondary lesions of the skin.

FIG. 31. Multiple cutaneous infiltrations on the right front and hind feet showing some loss of hair and various degrees of necrosis and ulceration. The hair has been clipped but not shaved.

FIG. 32. The scrotum of the same animal and the lesions which developed following inoculation. (See legend of Fig. 30.)

FIG. 33. A large crustaceous lesion over the elbow and paronychia of the fifth toe. Natural appearance.

PLATE 56.

FIG. 34. An early papular lesion on the posterior margin of the ear, showing the pale body of the lesion with a narrow zone of color at its base. Area shaved.

FIG. 35. A small papular lesion of a few days duration on the inner surface of the ear.

FIG. 36. Two small papules with central umbilication surrounded by a network of vessels. Lesions of only a few days duration.

FIG. 37. A group of small papular lesions showing circinate arrangement and bilateral symmetry.

PLATE 57.

FIG. 38. A fleshy papule of the upper eyelid.

FIG. 39. Multiple papular lesions of the upper lid and brow covered by heavy epithelial plaques, a transformation which frequently affects lesions such as that in Fig. 38.

FIG. 40. A fungus type of lesion which appears to be due to a continuous piling up of epithelial debris intermingled with a serous exudate. The body of this lesion was hardly more than 2 mm. in diameter.

FIG. 41. The same lesion at a later date showing a very irregular but still somewhat scaly surface. This figure is given to illustrate modifications which may take place in a given lesion.

FIG. 42. A macular erythema, or roseola, of the ear.

FIG. 43. Cutaneous infiltration following the fading of a roseola. There was still a distinct copper color in these areas.

PLATE 58.

FIGS. 44 to 47. Abnormalities of the skin frequently observed in rabbits infected with *Treponema pallidum* whose connection with the *pallidum* infection is still undetermined.

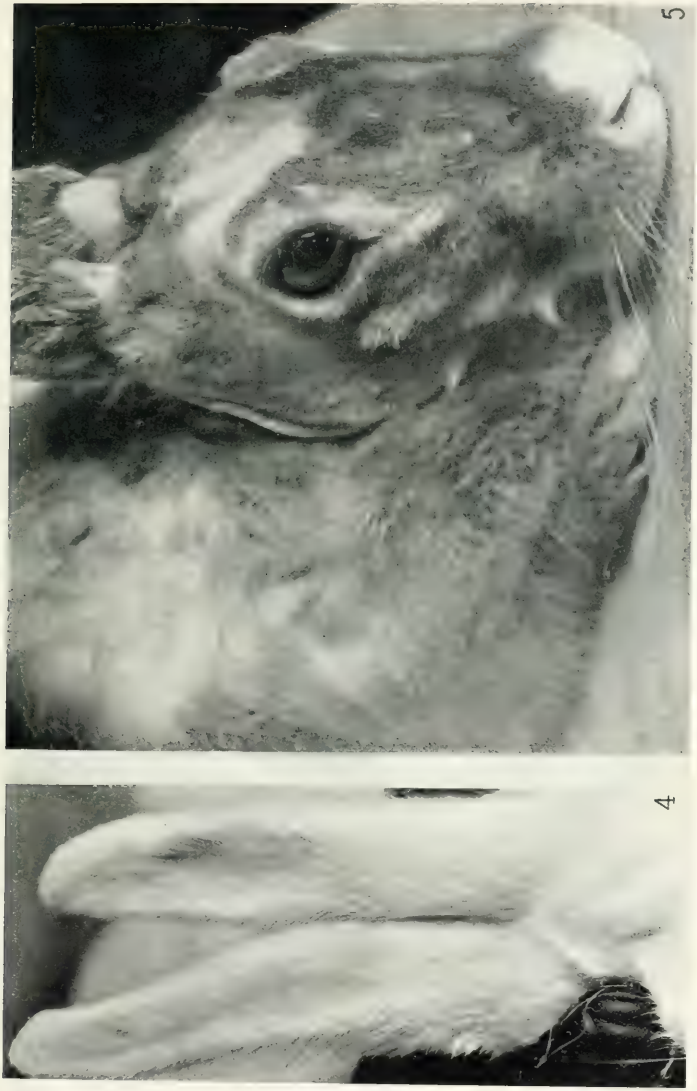
FIG. 44. An area of alopecia in the region of the loin showing irregular patches of thickening in the skin analogous to those which occur with a new growth of hair. These conditions are especially frequent in infected animals.

FIG. 45. Multiple follicular lesions over the cheek and neck. These lesions develop about the roots of certain hairs, and while they are quite common in infected rabbits, they are occasionally seen in apparently normal animals. Hair clipped.

FIG. 46. Alopecia of the cheek and pale circles in the skin occurring with the healing of lesions such as those in Fig. 45.

FIG. 47. A bare area of skin showing several minute crusts with a bristle protruding and numerous irregular areas of thickening and mottling in the skin. The one marked by the arrow was a definite syphilitic papule. The upper part of the area was shaved about 7 weeks before this photograph was taken but there had been practically no growth of hair within this time, which is most unusual.

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)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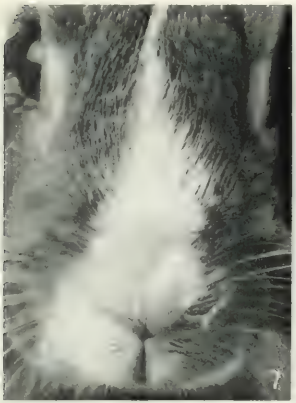
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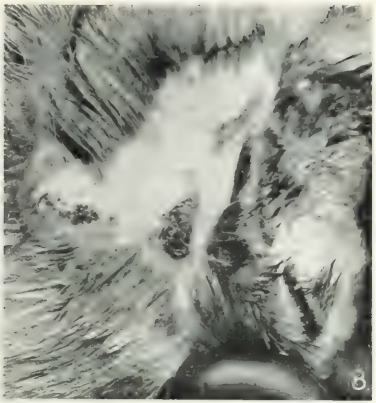
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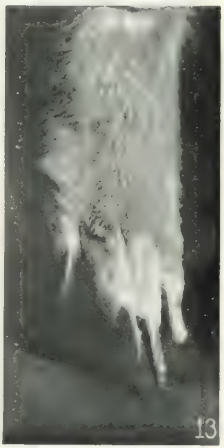
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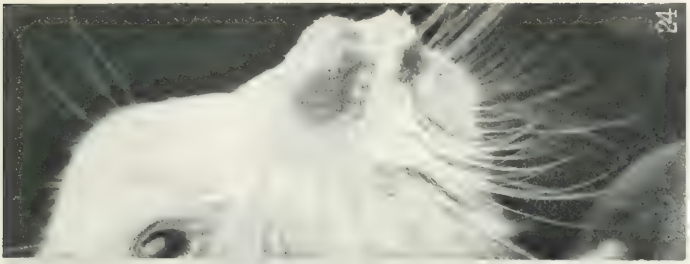
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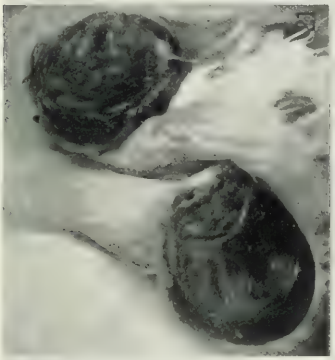

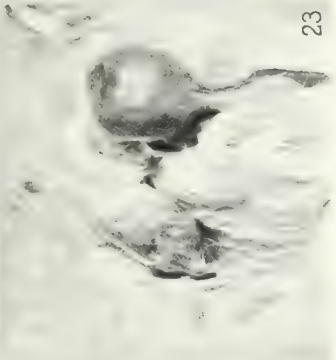
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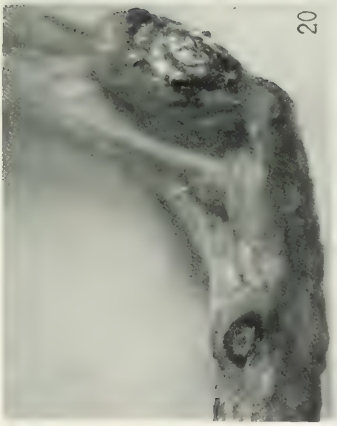
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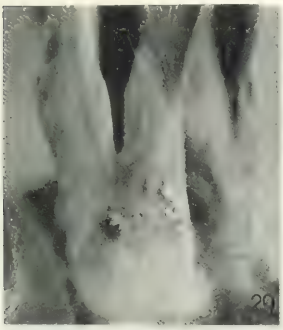

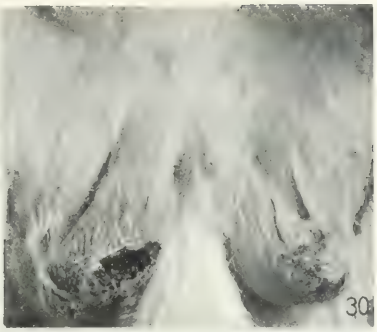

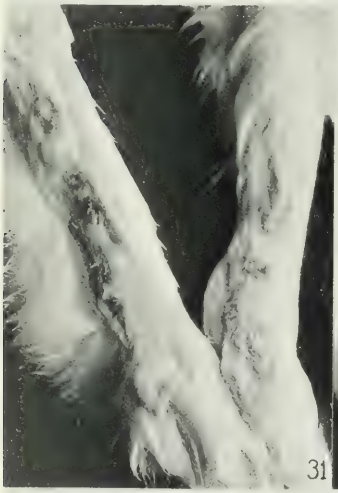

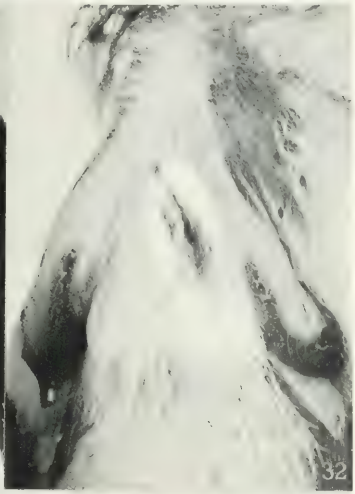

(Brown and Pearce: Experimental lesions in the rabbit. IV.)

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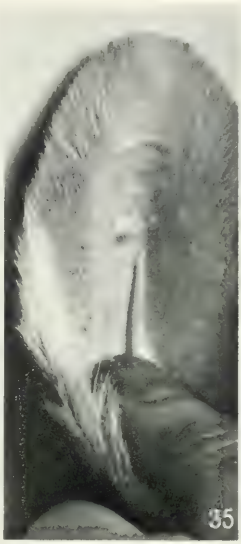
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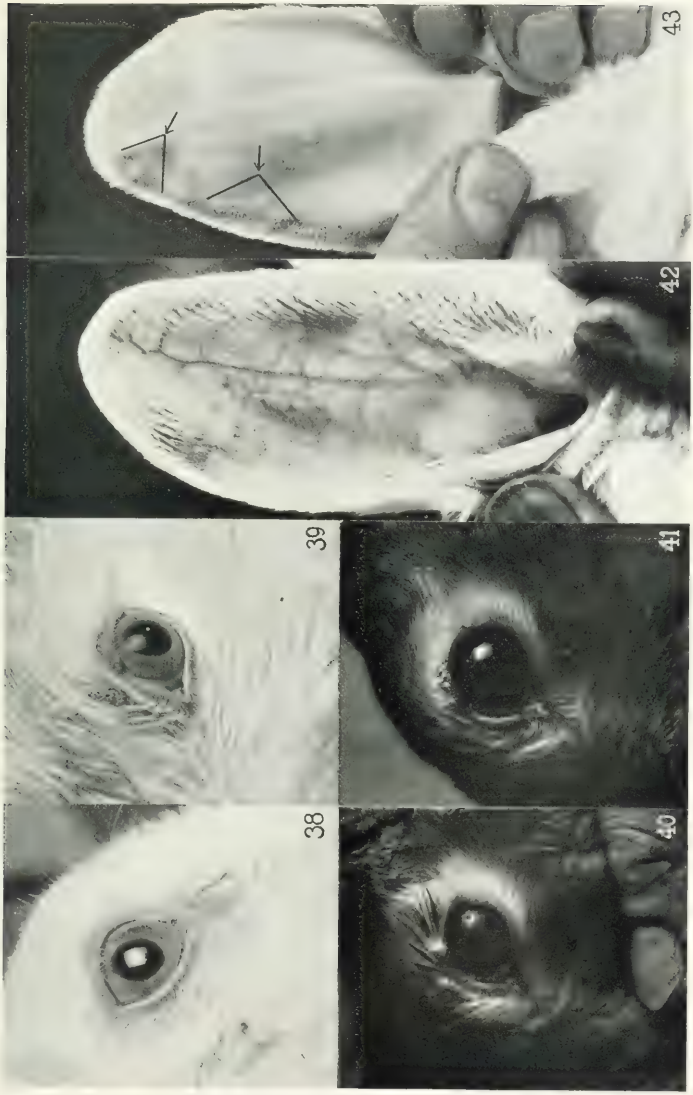
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EXPERIMENTAL SYPHILIS IN THE RABBIT.

IV. Cutaneous Syphilis.

Part 2. Clinical Aspects of Cutaneous Syphilis.

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Plates 59 to 77.

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The character of the cutaneous affections which have been described following local inoculations of *Treponema pallidum* in the rabbit is sufficient to identify these conditions as manifestations of a syphilitic infection and to throw some light upon processes of reaction in the experimental animal. However, the lesions are but the objective signs or the elements of the cutaneous infection, and it will be necessary to give some account of the clinical history of these conditions before a definite place can be assigned to them as integral parts of a generalized infection.

It is the purpose of this paper, therefore, to present such facts concerning the occurrence, distribution, and fate of cutaneous lesions as will enable one to formulate a general conception of cutaneous syphilis in relation to other phases of the experimental infection. In following out this plan of presentation, detailed statements of quantitative relationships will be omitted for the sake of simplicity as well as to avoid any prejudice which might be created from an attempt to assign such values upon the basis of the material which is at present available. Obviously, this aspect of the subject is of importance in itself and will be considered in due time.

Character and Distribution of Cutaneous Lesions in Different Parts of the Body.

From the description of cutaneous lesions which has been given, it is perhaps apparent that these affections tended to preserve a cer-

tain order of distribution and that the character of the lesions present differed somewhat according to their location.

The parts most frequently involved were the hind feet and legs, the head, the front feet and legs, and the tail.[1] This order of distribution was subject, however, to rather wide variations which corresponded in a measure with similar fluctuations in the incidence of different types of cutaneous affections. This is a fact of some importance, since the distribution of the lesions or the character of the lesions present in a given case was undoubtedly influenced to some extent by the particular organism with which the animal was infected. Further than this, the lesions did not occur indiscriminately over those parts of the body which have been mentioned but were confined, for the most part, to a few selected areas.

Lesions of the Head.

Among the animals studied by us, there was a large number which showed involvement of the skin about the head, and the lesions seen in this locality represented practically every form of cutaneous affection which has been described but with a decided predominance of processes of an infiltrative character. The points of especial predilection were certain parts of the face, the brows, the lids, the lips, the base and free portions of the ears, and the cheeks, and the affections peculiar to these areas will be taken up in the order given.

Face.—The lesions which occurred in the skin extending from the nose up over the forehead were chiefly small flattened areas of infiltration or slightly elevated papules, the usual location of which was the sides and bridge of the nose. The appearance presented by animals with affections of this type and the general distribution of the lesions may be illustrated by the photographs reproduced in Figs. 1 to 8. Some of these conditions were so apparent as to be recognized at a glance, but not infrequently there were no visible signs of abnormality and lesions could be detected only by careful palpation of the parts. Thus, the papule on the nose of the animal in Fig. 5 was made conspicuous by its location on a prominent part of the nose and in an area where the hair is short, while a lesion of essentially the same character in Fig. 6 gave no visible sign of its presence.

[1] Lesions about the anus and sheath were not included among the cutaneous affections but with those of mucocutaneous borders, while perineal affections were entirely eliminated for reasons which will be stated below.

As a rule, facial lesions were multiple, bilateral, and symmetrical. The more common ones were of the type represented in Figs. 2, 4, 5, and 6. Occasionally, however, affections of a more pronounced character were seen such as those in Figs. 1, 7, and 8. These were usually single and occupied a position at or near the midline in the region of the bridge or sides of the nose.

Brows.—The brows were less often involved than the face but were the seat of a variety of lesions. Those which appeared to be most characteristic of this area were small indurated papules of the type shown in Figs. 9 and 14, together with focal areas of infiltration and necrosis identical with those of the face. Two such lesions are indistinctly seen in Fig. 4. In addition to these, papules of a larger type or even granulomatous lesions were observed in a few animals, a characteristic example of which is given in Fig. 10.

With two exceptions, the lesions which occurred on the brows were bilateral and were usually accompanied by similar conditions on the upper eyelids (Fig. 14).

Eyelids.—Localized infections of the eyelids, while comparatively common occurrences in the rabbit, gave rise to two groups of conditions which could not be clearly separated from one another. In some instances, the lesions arose from the cutaneous surfaces of the lids and appeared to represent affections of essentially the same status as those of the brows; in others, they were clearly marginal in origin and related to the transitional area, or the infection was confined to the conjunctival surface.

However, the general character of the conditions found upon the external surfaces of the lids may be described without attempting to make any sharp distinction as to their origin.

The simplest form of lesion on both the upper and lower lids was a small papular infiltration of the type shown in Fig. 11, and these were especially common in the marginal areas. Fleshy papules measuring several millimeters in diameter were occasionally seen on the upper lids also (Fig. 13), but the usual affection was a small indurated papule with surface ulceration (Fig. 12) or lesions of a papulosquamous type such as those in Fig. 14. These lesions were often bilateral and multiple and were not infrequently associated with similar affections of the brows.

The distinctive feature of the lesions on the lower lids was their size. As a rule, they were larger and of a more fleshy character than those of the upper lids and exhibited a constant tendency to encroach upon the free margin of the lid. Their surface was usually scaly or covered with small crusts (Figs. 15 and 16), and in two instances, large chancre-like lesions were formed which underwent central necrosis and ulceration as shown in Fig. 17.

Lesions of the lower lid were all unilateral and single.

Lips.—There were comparatively few animals which showed localized infections on the lips, with the exception of those in which the lesions were situated along the margins of the nasolabial folds. The only conditions seen were patches of infiltration analogous to those in Fig. 18 or small indurated papules of the

type shown in Figs. 5 and 6. Other conditions affecting the marginal area will be described elsewhere.

The lower lip and chin were very rarely affected. Thus far, we have seen only three animals with lesions in this location, and all of these were comparatively small papules or areas of infiltration one of which is illustrated in Fig. 19.

Base of the Ears.—The skin at the base of the ears and over adjacent parts of the cheeks and neck formed another area for the localization of cutaneous affections (Figs. 20 to 25 and 83 to 88[2]). The lesions seen here were of two very different types and included processes varying from small papular infiltrations analogous to those in Fig. 20 to larger lesions of a circumscribed or diffuse character, (Figs. 21 to 25). In a few instances, large granulomatous masses such as those in Fig. 23 were seen, but the more common affections were small indurated papules or patches of diffuse infiltrations showing various forms of secondary alteration such as were described and illustrated in Part 1 of this paper. Not infrequently these were associated with similar processes on other parts of the ears or tended to spread from the basal area to adjacent parts of the ears as in Fig. 24.

By reference to the photographs illustrating these conditions, it will be seen that the position of greatest predilection for these affections was the lateral surface of the ears immediately below and at the sides of the intertragal incision. The lesions in this area were usually multiple; in some instances they were confined to one side, but in others they exhibited the most perfect bilateral symmetry (Fig. 23).

Ears.—The auricle was also the seat of cutaneous lesions in a number of animals. In the main, these were of the type of macular or small papular eruptions. The macular lesions have been described in detail and no further comment need be made here.

Upon the outer surface of the ears (Figs. 22 and 24 to 27), comparatively few lesions were seen, and, with the exception of that shown in Fig. 26, they were all situated on the lower portion of the ear and occupied one of four positions: the margins of the intertragal incision (Figs. 24 and 27), the medial surface of the ear (Fig. 25), and the area of the anterior or posterior marginal vessels (Fig. 22).

The majority of these lesions were flattened areas of infiltration covered by silvery or grayish yellow scales with occasional points of necrosis or ulceration covered by crusts; in a few instances they were small indurated papules.

Over the internal surfaces, three types of lesions were noted (Figs. 28 to 31). One of these was the macular erythema (Fig. 31) and another the small papular eruption (Fig. 30) which have already been described. These affections occurred chiefly upon the outer part of the ear and more often near the anterior than the posterior margin. They occurred singly, as in Fig. 28, or in groups, and occasionally exhibited a circinate arrangement or appeared to follow the course of the marginal vessels.

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[2] See Part 1 of this paper also: *J. Exp. Med.*, 1920, xxxii, 445.

A third type of lesion which was seen only once was that shown in Fig. 29. This condition developed as a flattened area of infiltration with slightly elevated margins. The surface was first covered with epithelial scales and later by yellowish gray crusts as shown in the photograph. There was a single lesion of this type on each ear.

Individual lesions on both the internal and external surfaces of the ears varied from a few millimeters to a centimeter or more in diameter and were usually symmetrically placed upon the two ears.

Cheeks and Neck.—With the exception of the area at the base of the ears, there were few lesions seen on the cheeks and neck which could be identified as syphilitic. The small follicular lesions and their various modifications referred to in the preceding paper (Part 1) were the most common conditions observed. In addition, several animals showed a diffuse infiltration of the skin over the cheeks and sides of the neck with a loss of hair and a tendency to desquamation or exfoliation.[3] There was one animal in which the area of involvement extended well down over the shoulder and resulted in the formation of two irregular patches of necrosis.[4] These were the only definite lesions seen in this part of the body. One other animal showed a small flattened lesion of a scaly character in the triangular area at the back of the neck which was probably specific, since at the same time there was a profusion of definite skin lesions elsewhere.

Lesions of the Hind Feet and Legs.

The hind feet and legs of the rabbit hold the distinction of being the most common location of cutaneous lesions (Figs. 32 to 55). This applied especially to granulomatous lesions, and it was here that this type of affection reached its highest state of development. In addition to the granulomata, there were, however, rashes of various types, onychias, and paronychias.

Ordinarily, these affections produced but little change in the general appearance of the parts, and the conditions which one might note were in the main prominent swellings due to the presence of large granulomatous lesions or areas of necrosis and ulceration such as are shown in Figs. 32 to 34. Instances of this kind were comparatively rare, however, and as may be seen by reference to Figs. 35 to 38, which are photographs taken before and after removal of the hair, even marked affections of the skin usually produced only slight changes in appearance.

[3] See the section on alopecia in Part 1 of this paper.

[4] Fig. 8, Part 1.

The lesions on the hind feet and legs were generally located along the lateral margins of the feet more upon the dorsal than the plantar surface, and were distributed from the region of the tendo achillis to the base of the fifth toe. The positions of greatest frequency were the region of the tarsus and external malleolus, the base or tuberosity of the so called fifth metatarsal, the lateral and posterior surfaces of the heel and tendo achillis, and the region of the metatarsophalangeal joint. These peculiarities of distribution and the character of the lesions found in the various locations are brought out by the photographs reproduced in Figs. 36 to 55. It will be noted that the positions enumerated are in a sense pressure points or are skin areas overlying bony or tendinous prominences, and a very large proportion of the cutaneous lesions of the hind feet and legs occurred at these particular points.

Typical examples of the conditions described are illustrated in Figs. 35 to 38 and 39 and 40 which show the hind feet of two animals with marked skin involvement. By reference to the other figures of the series (Figs. 41 to 55), it will be seen that individual variations in the size, number, and location of the lesions were very great. In some instances, frank lesions of the dorsum of the foot or ankle were noted (Figs. 36, 37, and 50), and in their growth those which developed upon the sides of the feet extended in this direction rather than towards the plantar surface. However, plantar lesions or lesions which extended towards the plantar surface were also encountered in some instances (Figs. 51 to 53), and were usually located in the tarsal arch or more exactly in the cushion covering the head of the os calcis just posterior to the base of the fifth metatarsal. Lesions occupying this position are shown in Figs. 49, 51, and 52.

Comparatively few lesions were seen on the legs, and most of these were situated over the tendo achillis, usually not more than a centimeter or so above its attachment to the calcaneus. Occasionally, however, they occupied a much higher position, as in Fig. 46, and in two instances lesions were found on the anterior surface of the shins. One of these is shown in Fig. 36.

The characteristics of distribution described were more closely observed by granulomatous lesions than by those of an infiltrative character. The latter manifested a greater tendency to spread in a linear fashion along the outer side of the foot and might, as in Fig. 54, form an almost continuous line of lesions extending from ankle or heel to the toe, but even here the tendency to localize or to concentrate at certain points was still apparent (Figs. 53 to 55).

As may be gathered from an examination of the accompanying illustrations, lesions of the hind feet and legs were occasionally single

or multiple and unilateral, but in the vast majority of cases they were multiple, bilateral, and symmetrical in their distribution.

After what has been said in regard to similar affections elsewhere, no description of the lesions seems necessary. The illustrations are sufficient in themselves to convey the necessary impressions of the variations in size and character of the several types of lesions to enable one to form a proper conception of the subject.

In addition to the lesions described, mention may be made of the occasional presence of such conditions as onychia and paronychia.

Lesions of the Front Feet and Legs.

The prevailing lesions of the front feet and legs were of essentially the same character as those of the posterior extremities but with a larger proportion of infiltrative processes. The common location of these affections was the extensor and lateral surfaces of the fore arms and the dorsum and sides of the feet as shown in Figs. 56 to 70.

The granulomata were usually situated on the fore arms just above the carpus or on the lateral margins from the level of the carpus to the base of the fifth toe; small multiple lesions were sometimes distributed over the carpus and dorsum of the feet as in Figs. 62 and 65. Occasionally also granulomatous lesions were found at a much higher level on the fore arm (Fig. 64), and in rare instances were located on the flexor instead of the extensor surface (Fig. 59).

The general distribution of the cutaneous infiltrations was much the same as that of the granulomata, but they differed in certain respects. In the case of the infiltrations, the lesions usually appeared along the ulnar margins of the fore arm and carpus instead of the extensor surfaces; they almost invariably extended over the dorsum and sides of the feet (Figs. 66 to 73) and frequently involved the median as well as the lateral surfaces (Figs. 69 and 71). In one instance, large crustaceous lesions were formed about the elbow (Figs. 74 and 75).

The cutaneous granulomata not infrequently appeared as single lesions of a large size or as multiple affections of one fore arm. In contrast to this, the infiltrations were in all cases multiple affections and bilaterally symmetrical in their distribution.

Other conditions affecting the fore paws were onychia and paronychia which were most common on the outer or fifth toe (Figs. 74 and 75), but in several animals other toes as well were involved (Fig. 71).

In connection with lesions of the fore arms, attention may be called to the frequent occurrence of edema during periods of marked activity on the part of

various types of cutaneous lesions, since examples of this condition may be seen by reference to Figs. 59 and 61. In the first of these, a very pronounced swelling about the carpus and foot can be made out, and in Fig. 61, there is a marked edema of the skin and subcutaneous tissues which extends beyond the middle of the fore arm.

In other instances where no edema was present, it was noted that shaving an area where there was syphilitic involvement or the infliction of slight trauma by other means which had no effect upon the skin of a normal animal was frequently sufficient to cause a rapid development of edema which lasted sometimes for days. This urticarial reaction of the skin, if it may be termed such, was not peculiar to cases of cutaneous infection but occurred also in animals in which the lesions were situated in the deeper structures, as in the bones. It suggests, therefore, the action of some toxic influence upon the vascular mechanism of parts adjacent to foci of active syphilitic infection, and this feature of the infection is worthy of consideration in connection with the various cutaneous and other reactions which occur subsequent to the administration of therapeutic agents.

Lesions of the Tail.

Localized infections of the tail form a group of conditions which is somewhat obscure. Uhlenhuth and Mulzer (1) reported "tail tumors" as a frequent occurrence following generalized inoculation of rabbits, and the condition thus described was in most instances a bulbous swelling of the distal end of the tail. Except in this connection, we have seen no mention of tail lesions in the literature. Such conditions do occur, however, and are probably of rather frequent occurrence, but the difficulties surrounding an examination of this part of the body are so great that we were unable to give it the attention which it probably deserved and hesitate, therefore, to commit ourselves as to the frequency and importance of this class of affections.

Again, we have been able to recognize two conditions affecting the skin of the tail (Figs. 76 to 82). The one most commonly observed was a granulomatous lesion generally involving the ventral surface or sides of the tail, rarely the dorsal (Figs. 76 to 81). As may be seen in the accompanying illustrations, these lesions were either single or multiple, and while they occurred at various levels, they were

more often found on the proximal and middle thirds than towards the outer end of the tail.

The second type of affection was that shown in Fig. 82. In these cases, the skin involvement was of an equally marked character, especially towards the end of the tail, but partook more of the nature of a diffuse infiltration. The photograph reproduced in Fig. 82 shows a thinning of the hair and the presence of bald patches over the ventral surface of the tail which characterized this group of affections. Towards the outer end, there is also a small area of necrosis. In one case of this kind, the skin covering the entire tail underwent necrosis and sloughed away.

The six cases of cutaneous syphilis of the tail which have been used for illustration are such as might be recognized without any considerable difficulty, and it will be noted that none of them conforms to the "tail tumor" type of lesion. Bulbous expansions of the end of the tail were observed, however, in a few animals but were always of a relatively slight degree and were difficult to detect because of the fact that the physical alterations present were within the range of normal variations in tail structure. These variations are so great that unless one has an accurate record of the tail of each animal before inoculation or the pathological alterations which take place are quite marked, little can be determined by palpation and the fur must be removed before inspection will be of any material assistance. These procedures we were unable to carry out as a routine part of our work and we are not in a position, therefore, to speak with any degree of assurance as to conditions other than those observed.

Lesions of the Trunk.

With the exception of the perineum, few instances of cutaneous lesions involving the trunk have come under our observation. As previously stated, alopecia was noted in a number of animals, and after removal of the hair from localized areas some peculiar conditions were observed which may or may not have been syphilitic.[2] This is not to be interpreted as evidence that this portion of the body remains uninvolved. On the contrary, it is known that lesions may occur upon the trunk, since a pronounced eruption on the back was one of the conditions described by Grouven (2) in the first case of generalized syphilis reported. It does mean, however, that under

ordinary circumstances, marked involvement such as one frequently observes in other parts of the body is far less common on the trunk. It should be pointed out also that the regions in which minor lesions were most frequently observed were those which were most accessible to examination, and when it is recalled that some of these lesions are extremely inconspicuous, it seems not unlikely that many such affections might escape detection altogether if situated upon the more thickly covered portion of the body. The occurrence of cutaneous lesions of the trunk must be regarded, therefore, as problematical and a field for future investigation.

As regards the perineum, it may be said that lesions in this locality, including those of the genitalia as well as the surrounding skin surfaces, were quite numerous and included two classes of affections, one representing conditions of cutaneous origin and the other affections of the mucocutaneous borders.

The first of these groups comprised lesions which for the most part were closely connected with the seat of inoculation, and we have avoided placing too much stress upon them lest some confusion might arise between extensions from primary lesions and generalization of the infection as it is commonly understood. However, the descriptions of scrotal lesions given in the preceding papers (3–6) will apply equally well to those which arise in this area as a result of generalization of the virus. Other lesions of the perineum will be described in connection with affections of mucocutaneous borders.

Clinical History of Cutaneous Lesions.

An exact statement of the time of occurrence, the duration, and the relative frequency of different types of cutaneous lesions in the rabbit can hardly be given without entering into a detailed analysis of the experimental conditions under which the lesions developed, and must be deferred, therefore, until the subject can be approached from this standpoint. There are, however, certain facts concerning the clinical history of these affections which may be recorded, some of them in greater detail than others.

Cutaneous Eruptions.—The cutaneous eruption in the rabbits usually consisted of only a few lesions occurring singly or in small

groups upon some one part of the body such as the head or the feet and legs. Occasionally, the lesions were more numerous and more widely distributed, several parts of the body being involved at about the same time or in rapid succession to one another.

The order in which the lesions appeared was somewhat irregular. It was not uncommon for several or all of them to make their appearance at about the same time. In other instances, one or two lesions developed, and no others were detected for a week or more when new lesions appeared and were followed by others at rather long and irregular intervals which at times extended over several months, or, after the appearance of the first lesions, others developed at short intervals until the eruption was complete.

How many distinct periods of eruption might occur is impossible to say, since the majority of the animals were not held for any great length of time. Among those which were kept under observation for a year or more, some showed only a single period of cutaneous eruption, while in others, there were several such periods separated by long or short intervals during which no lesions were present or no new lesions appeared.

In some instances, the consecutive eruptions consisted of lesions situated in places not previously involved, while in others, they might be regarded more as relapses than as new eruptions, since the new lesions developed at the site of older ones.

It was usually but not invariably the case that the lesions of the first eruption were of a larger type and more numerous than those of succeeding crops. This applied especially to the granulomata, an instance of which may be seen by comparing Figs. 39 and 40 with Figs. 41 and 42. Figs. 39 and 40 represent the first cutaneous eruption on the hind feet. These lesions healed completely and remained healed for between 4 and 5 months at which time two of them recurred (Figs. 41 and 42).

Another very important feature of the cutaneous eruption was the apparent influence exerted by one lesion or group of lesions upon another. This was best shown in instances where multiple lesions occurred in the same locality, as upon the fore arms and wrists of the animal in Figs. 62 and 63. When, as in this case, a number of lesions made their appearance at about the same time, the growth of most of

them was abortive, and only one or two developed to any considerable extent. It may be recalled that this same condition was pointed out in connection with the development of multiple lesions of the scrotum.

The inhibition thus exerted by one focus of reaction upon another is a phenomenon of the most fundamental importance and furnishes the key to an explanation of many conditions which characterize the experimental infection. In the present instance, it probably accounts for the existence of so few lesions and the unusual size which individual lesions frequently attain.

From this description of cutaneous eruptions, it is apparent that they were exceedingly variable as to the number and character of the lesions present at a given time, the manner of their appearance, and the number of eruptions which might occur in a given case.

Time of Occurrence of Cutaneous Lesions.—Cutaneous lesions were among the earliest manifestations of a generalized infection, but the time of their appearance was subject to very wide variations. This appeared, however, to be partly due to differences in experimental conditions. The first cutaneous eruption usually appeared between the 2nd and 4th months after inoculation and on the whole was earlier in the case of the granulomata than with lesions of an infiltrative character. The earliest recorded time for the appearance of cutaneous lesions was 3 weeks after inoculation, and the longest interval between inoculation and the first cutaneous eruption was 2 years and 8 months; the next longest was between 6 and 7 months. It may be said, therefore, that the first cutaneous eruption appeared at from 6 weeks to 6 months after inoculation with occasional cases occurring earlier or later, depending upon the conditions of the infection.

As regards the occurrence of subsequent eruptions, or the interval between eruptions, very little can be said. In the few animals which were held for any considerable length of time, the period of active eruption rarely extended beyond 4 to 6 months after inoculation. One case has been mentioned, however, in which the first lesions were noted 2 years and 8 months after inoculation. A second eruption occurred in this animal 3 years and 5 months after inoculation, and a third eruption 2 months later. Lesions of this second group are shown in Fig. 52.

Two other animals of our series showed repeated periods of active cutaneous eruption extending over more than 2 years from the time of inoculation, but during this time they were never entirely free from lesions. A third animal now under observation has followed much the same course for a period of 8 months. In a fourth animal, as in the case of the one cited above, there were three sharply separated periods of eruption spaced at intervals of approximately 7 months. These few cases will serve to indicate the difficulty in attempting to set any time limits upon the occurrence of cutaneous lesions when so few animals were kept under observation for any considerable period of time, but they also indicate that cutaneous affections may occur during the late as well as the early stages of the infection.

Clinical Course of Cutaneous Lesions.—The clinical course of the cutaneous infection was essentially the same as that of the testicular or scrotal lesions. The tendency to pursue a periodic or relapsing course was apparent in the development and resolution of individual lesions as well as in the recurrence of healed lesions and the development of successive crops of eruptions.

These features of the skin reaction were again most evident in the granulomata, the growth of which usually proceeded by irregular stages interrupted by periods of inaction or even regression. Relapse of partially or completely healed lesions, which is but an exaggerated form of this reaction, was a comparatively common occurrence and may be illustrated by the series of photographs reproduced in Figs. 83 to 88 representing the state of a lesion at intervals of 90, 97, 105, 133, 141, and 160 days respectively after inoculation. This type of phenomenon was practically constant, but we have little evidence upon which to base an estimate of the frequency of relapse of completely healed lesions. It may be mentioned, however, that of the few animals kept under observation for a year or more, a number showed recurrence, and with three of them there were several periods of complete healing of individual lesions followed by relapse.

Duration of Cutaneous Lesions.—Spontaneous regression and healing were the common fate of all cutaneous lesions, but great differences were found in the duration of different types of lesions as well as of individual lesions. As was mentioned elsewhere, the macular erythemata were characteristically of short duration, sometimes dis-

appearing within 24 to 48 hours and rarely persisting for more than a few days. In like manner, small infiltrations not infrequently disappeared spontaneously within a few weeks after they were first noted.

On the other hand, granulomatous lesions and many of the infiltrations, especially the papular lesions of the brows and lids, were more enduring as a rule. For example, the papular lesions of the brows and lids shown in Fig. 14 lasted more than a year before they completely disappeared, and the lesion at the base of the toe in Fig. 48 is one of a small group which showed an even greater persistence, having lasted for more than 2 years. These were exceptional cases, however, and from the data available, it would appear that the average duration of cutaneous lesions was hardly more than 2 to 4 months, although in a fair percentage of animals, this period might be prolonged by several months.

The duration of active skin infection was somewhat longer than that of the individual lesion, since all the lesions present did not pursue a parallel course of changes. The available data bearing upon this feature of the infection, however, are insufficient to permit of an estimation of the time which such infections might endure.

Detection and Diagnosis of Cutaneous Lesions.

In view of the great frequency with which cutaneous lesions appear to follow local inoculations of *Treponema pallidum* in the rabbit and the fact that so few of these conditions have been described, it seems well to refer briefly to the detection and diagnosis of this class of affections.

The first essential to the detection and diagnosis of cutaneous lesions is obviously a knowledge of the character and distribution of these affections, and this we have attempted to supply. Large granulomatous lesions such as those in Figs. 32 to 34 which produce striking irregularities in the contour of the affected parts, or lesions which have resulted in a considerable destruction of the skin surface, as in Figs. 74 and 75, may be seen almost at a glance if one knows where to look for them. Prominent swellings and surface erosions are not always present, however, and at all events are late phenomena. The great majority of the lesions which affect the feet, legs, and tail are

concealed from view and can be detected only by palpation, rarely by inspection. Lesions about the head are more exposed as a rule, and careful inspection of the regions in which they are known to occur is highly essential, but with the exception of a few areas such as the ears and the eyelids, even here the chief reliance for the early detection of cutaneous lesions must be placed upon palpation.

The technique of this operation consists in picking up the skin and gently rolling it between the thumb and finger. In this way, one is soon capable of detecting the slightest thickening or irregularity in the skin, and if the hair is removed and these initial changes are carefully followed from day to day, little difficulty will be experienced in arriving at a diagnosis.

If the animals are separately caged and well cared for, few conditions will arise which are apt to be confused with typical syphilitic lesions, even the smaller infiltrations and erosions. Apart from diseases of the skin, such as mange, and traumatism inflicted by the animal itself, which are very readily recognized, the only three conditions which need be considered are small focal abscesses, old scars, and focal thickenings due to a new growth of hair. The first two conditions are, in the main, trunk affections and should not be confused with syphilitic lesions. The third condition may occur anywhere and is especially troublesome about the face, but clipping of the hair and careful observation will usually enable one to arrive at a correct diagnosis. Examination for spirochetes may or may not prove helpful in these cases and may even defeat the object of the examination by causing regression of a syphilitic lesion before a characteristic condition has been established.

CONCLUSIONS.

The description of cutaneous syphilis in the rabbit following local inoculation is incomplete in many respects but is sufficient to indicate that a generalized infection of the skin does occur in a large number of animals—just how often and under what circumstances we shall not attempt to say.

It is apparent, however, that the cutaneous affection is a very characteristic one. The lesions themselves bear the marks of a varied

but definite pathological process identical in all respects with the reaction set up in the scrotum by local inoculation of *Treponema pallidum*. The character of the lesions and, withal, their great multiformity, the time relations existing between inoculation and the appearance of the cutaneous lesions, the occurrence of successive crops of eruptions, the relapsing course of the disease, and the preservation of a fixed order of distribution of the lesions, are highly suggestive of cutaneous syphilis in man, and it is believed that as more is known of the experimental infection and with better adaptation of organisms, the analogy will become even closer.

SUMMARY.

From the study of a large number of rabbits with generalized cutaneous syphilis following local inoculation with *Treponema pallidum*, lesions were found most often about the hind feet and legs, the head, the front feet and legs, and the tail. There was further evidence of a selective distribution of cutaneous lesions in the fact that, on a given part of the body, the lesions were usually confined to a few restricted areas. About the head, they occurred almost exclusively on the sides and bridge of the nose, the lids, the brows, the lips, and the base and free portions of the ears. On the front feet and legs, the seat of predilection was the extensor and lateral surfaces of the fore arm, the carpus, and the feet, while on the posterior extremities they were situated upon the dorsum and lateral surfaces of the feet and ankles from the level of the tendo achillis to the base of the fifth toe. The positions of greatest frequency were the region of the tarsus and external malleolus, the base of the fifth metatarsal, the lateral and posterior surfaces of the heel and tendo achillis, and the base of the fifth toe. In many instances, the positions of predilection were exposed positions or areas of skin covering bony or tendinous prominences.

It was also found that the character of the lesions differed somewhat in the various locations. The lesions of the head were mostly small circumscribed papules or processes of diffuse infiltration; on the fore arms and feet, affections of this type were about equally divided with larger granulomatous masses of a chancre-like character, while on

the hind feet and legs, granulomatous lesions were far more numerous than those of any other type and frequently reached a very large size.

The cutaneous eruption usually consisted of only a few lesions confined to some one part of the body, but occasionally they were more numerous and more widely distributed. In this connection, it was noted that when multiple lesions appeared in a given area at about the same time, the growth of most of them was abortive, and, as a rule, only one or two developed to any considerable size. Especial emphasis was placed upon this phenomenon of inhibition as a factor of fundamental importance in the experimental infection.

From clinical observation, it was found that, as a rule, the first cutaneous eruption occurred at from 2 to 4 months after inoculation but might occur either earlier or later, depending upon the circumstances in the individual case. The earliest eruptions appeared 3 weeks after inoculation and the latest 2 years and 8 months, but, as a rule, the time between inoculation and the appearance of the first eruption did not exceed 4 to 6 months.

Successive crops of cutaneous lesions appeared in a number of animals usually within the first 6 months after inoculation. In a few instances, however, there were repeated eruptions extending over a period of 2 years or more, the longest recorded period being 3 years and 7 months.

The duration of individual lesions was found to be extremely variable, ranging from a few days in the case of a macular erythema to more than 2 years in the case of a few granulomatous lesions. The average duration of the lesions appeared to vary somewhat with the nature of the lesion but on the whole was not more than 2 to 4 months. No limits could be fixed, however, for the duration of an active skin infection as a whole.

Again, it was found that the cutaneous infection tended to pursue a periodic or relapsing course. This was seen in the mode of growth and resolution of individual lesions, the occurrence of successive periods of eruption, and the recurrence of completely healed lesions, all of which was interpreted as evidence of the essential relapsing nature of syphilitic infections.

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EXPLANATION OF PLATES.

With the exception of Fig. 31 the illustrations are from unretouched photographs which represent the objects at their natural size. The time given is estimated from the date of inoculation unless otherwise stated.

PLATE 59.

FIGS. 1 to 4. Clinical appearance of animals with syphilitic lesions about the face.

FIG. 1. 58 days. An extensive infiltration of the frontal region showing loss of hair and exfoliation over the affected area.

FIG. 2. 138 days. Multiple foci of infiltration and necrosis distributed over the sides and bridge of the nose. There was a similar condition on the left side of the face.

FIG. 3. 249 days. A diffuse area of infiltration with some loss of hair and desquamation of epithelium involving a portion of the lip and the skin over the side of the nose.

FIG. 4. 188 days. Multiple focal lesions over the lower portion of the nose, the right upper lip, and the brows. Lesions were also present about the nares.

PLATE 60.

FIGS. 5 to 10. Syphilitic lesions of the face and brows.

FIG. 5. 69 days. Early papular lesion on the side of the nose.

FIG. 6. 98 days. Small nodular lesion, side of the nose, which was not visible until the hair was removed.

FIG. 7. 128 days. Circumscribed nodular lesion with central necrosis, situated on the lower portion of the bridge of the nose.

FIG. 8. 169 days. Extension and transformation of the lesion in Fig. 7.

FIG. 9. 138 days. Small papular lesions with apical necrosis on the brow.

FIG. 10. 112 days. Large nodular syphilide with central necrosis and ulceration on right brow.

Plate 61.

Figs. 11 to 19. Syphilitic lesions of the lids, brows, and lips.

Fig. 11. 98 days. Early papular lesions on upper and lower lids. There are two small lesions on the margin of the upper lid and a single larger lesion on the lower lid.

Fig. 12. 108 days. Small indurated papule with surface ulceration on the margin of the upper lid.

Fig. 13. 134 days. Large papular syphilide, upper lid, with slight necrosis at the center and considerable desquamation of surface epithelium.

Fig. 14. 13 months. Multiple lesions of the upper lid and brow. These lesions were of the nature of small papular infiltrations covered by heavy epithelial scales.

Fig. 15. 80 days. Large nodular lesion, lower lid. This lesion was of an intense copper color with small patches of scales over its surface.

Fig. 16. 113 days. Nodular lesion of the lower lid similar to that in Fig. 15, showing foci of necrosis covered by crusts.

Fig. 17. 84 days. Large granulomatous lesion of the lower lid. The center is necrotic and covered by a thick crust.

Fig. 18. 65 days. Circumscribed area of infiltration, right upper lip. The surface of the lesion is necrotic and covered by a crust.

Fig. 19. 8 months. Multinodular area of infiltration on the chin. This lesion was of a deep copper color and showed several areas of exfoliation.

Plate 62.

Figs. 20 to 23. Syphilitic lesions of the ears, basal region.

Fig. 20. 53 days. Two small areas of infiltration in the skin marked by arrows. These lesions developed no further than the condition here shown.

Fig. 21. 113 days. Multiple nodules in the skin at the base of the ear.

Fig. 22. 101 days. Small circumscribed area of infiltration with central necrosis just below the intertragal incision. A second lesion is seen posterior to this and a third on the anterior surface of the ear. All these lesions showed a central area of ulceration and a marked tendency to accumulation of epithelial scales.

Fig. 23. 128 days. Large granulomatous lesions at the base of the ears showing bilateral symmetry of this class of affection. A few small areas of infiltration are also seen above the nodular lesions on the left ear.

Plate 63.

Figs. 24 to 29. Syphilitic lesions on the free portions of the ears.

Fig. 24. 8 months. The same animal as in Fig. 22. Spreading patches of infiltration on the lateral surface of the ear.

FIG. 25. 8 months. The same animal. Syphilitic lesions on the median surface of the ear. One of these was a very small area of infiltration (marked by arrow). The other shows an area of necrosis surrounded by a zone of infiltration.

FIG. 26. 104 days. Indurated nodular lesion on the anterior and outer surface of the ear.

FIG. 27. 13 months. Diffuse area of infiltration with exfoliation and necrosis spreading along the anterior margin of the ear.

FIG. 28. 60 days. Circumscribed nodular lesion on the inner surface of the ear, at an early stage of its development.

FIG. 29. 140 days. Circumscribed area of infiltration with raised edges and necrotic center situated on the anterior margin of the inner surface of the ear.

PLATE 64.

FIG. 30. 97 days. Multiple papular lesions on the inner surface of the ear showing circinate arrangement and bilateral symmetry.

FIG. 31. 132 days. Erythematous patches in the ears showing bilateral symmetry.

PLATE 65.

FIGS. 32 to 34. Clinical appearance presented by animals with large granulomatous lesions of the hind feet.

FIG. 32. 122 days. There are four large granulomatous nodules along the outer side of the right foot; on the left, there is a single large lesion in the region of the metatarsal and a second on the outer side of the heel.

FIG. 33. 122 days. Lateral view of the right foot of the same animal.

FIG. 34. 177 days. The same foot at a later date showing the appearance presented after extensive necrosis and ulceration had taken place.

PLATE 66.

FIGS. 35 to 38. Nodular or granulomatous lesions of the hind feet and legs.

FIG. 35. 69 days. Anteroposterior view before removal of the hair.

FIG. 36. 81 days. Appearance presented at a slightly later date with hair removed. The nodular swellings over the outer portions of the fifth metatarsals are due to periosteal lesions.

FIGS. 37 and 38. Lateral views of the same feet. Note especially the character and distribution of the lesions.

PLATE 67.

FIGS. 39 to 42. Nodular or granulomatous syphilides of the hind feet and legs.

FIGS. 39 and 40. 115 days. Right and left hind feet of the same animal showing the character and location of lesions and bilateral symmetry.

FIGS. 41 and 42. 10 months. Hind feet of the same animal showing recurrent lesions at the base of the fifth metatarsals. Note the relative size of the lesions as compared with the original lesions in Figs. 39 and 40.

PLATE 68.

FIGS. 43 to 46. Granulomatous lesions of the hind feet and legs.

FIGS. 43 and 44. 154 and 148 days respectively. Right and left hind feet of the same animal showing single chancre like lesions, one over the external malleolus and the other on the lateral and posterior surface of the tendo achillis just above the heel.

FIG. 45. 76 days. Granulomatous lesions over the tendo achillis, side of the heel, and base of the fifth metatarsal.

FIG. 46. 125 days. Large granulomatous lesion situated high up on the lateral and posterior surface of the tendo achillis.

PLATE 69.

FIGS. 47 to 50. Atypical distribution of granulomatous lesions on the hind feet.

FIGS. 47 and 48. 2 years and 2 months. The same animal. Single granulomatous lesions involving the heel of the left foot and the base of the fifth toe on the right. These lesions are the same as those shown in Figs. 32 to 34.

FIGS. 49 and 50. Left and right hind feet of the same animal.

FIG. 49. 91 days. There are four lesions on this foot, one over the external malleolus, a second on the plantar arch, the third on the dorsal surface of the fifth metatarsal, and the fourth at the base of the outer toe.

FIG. 50. 83 days. There are also four clearly defined lesions on this foot, all of which occupy positions on the dorsum of the foot, which is quite unusual.

PLATE 70.

FIGS. 51 to 55. Plantar lesions and lesions of an infiltrative character on the hind feet and legs.

FIG. 51. 75 days. Large granulomatous lesion on the outer and plantar surfaces of the tarsus and a smaller lesion over the tuberosity of the fifth metatarsal. There is a third lesion on the plantar surface at the base of the fifth toe.

FIG. 52. Lesions identical with those in Fig. 51. The point of chief interest is the time of their occurrence which was 3 years and 5 months after inoculation.

FIG. 53. 68 days. Discrete infiltrations over the lateral surface of the foot with a tendency towards a plantar position.

FIGS. 54 and 55. 15 months. Hind feet of the same animal. Multiple infiltrations distributed over the lateral margin of the foot. In Fig. 54, the lesions show necrosis, some exfoliation, and a tendency to fuse with one another.

PLATE 71.

FIGS. 56 and 57. Nodular or granulomatous lesions of the front feet and legs.

FIG. 56. 81 days. Multiple nodular lesions over the extensor and lateral surfaces of the fore arms and feet. Note character and location of the lesions.

FIG. 57. 69 days. The same feet at a slightly earlier period before removal of the hair.

PLATE 72.

FIGS. 58 to 61. Lateral views of the same animal as that in Figs. 56 and 57. Note the presence of a lesion on the flexor surface of the fore arm and the edematous swelling shown about the carpus and foot in Fig. 59 and extending to the middle of the fore arm in Fig. 61.

PLATE 73.

FIGS. 62 to 64. Nodular and granulomatous lesions of the fore arms and feet.

FIG. 62. 113 days. Early multinodular affection with lesions on the extensor surface of the fore arms, carpus, and dorsum of the feet.

FIG. 63. 128 days. Later stage in the development of the same lesions. Note the disappearance of most of the lesions shown in Fig. 62 and marked development of single lesions on each fore arm.

FIG. 64. 84 days. Large chancre-like lesion on the extensor surface of the fore arm situated at an unusually high level.

FIG. 65. 60 days. Early nodular syphilides, dorsum and lateral surface of the foot. The prominent swelling over the carpus was due to a periosteal lesion.

PLATE 74.

FIGS. 66 to 70. Infiltrative lesions on the fore arms and feet.

FIGS. 66 and 67. 202 days. Right and left front feet and legs of the same animal. The lesions here shown were in the beginning parchment-like areas of infiltration (Fig. 66) which underwent necrosis and ulceration (Fig. 67).

FIG. 68. 138 days. Appearance presented by an early lesion similar to those in Figs. 66 and 67. The presence of a lesion is suggested by partial loss of hair and discoloration of the skin shown on the lateral surface of the carpus.

FIG. 69. 157 days. Diffuse infiltration on fore arm, carpus, and dorsum of the feet. Hair clipped.

FIG. 70. 184 days. Lateral view of the lesion in Fig. 69, taken during a period of marked activity.

PLATE 75.

FIGS. 71 to 75. Cutaneous infiltrations, front feet and legs.

FIG. 71. 15 months. Frontal view of the feet to show lesions present over the median surface of the toes.

FIGS. 72 and 73. The same as Fig. 71, showing lesions on the lateral surface of the fore arm and at the base of the toes.

FIGS. 74 and 75. 8 months. There is an area of widespread infiltration over the elbows with surface necrosis, exfoliation, and the formation of weeping patches; there is also a well marked paronychia of the fifth toe on both sides.

PLATE 76.

FIGS. 76 to 82. Syphilitic lesions of the tail.

FIG. 76. 77 days. Early multiple granulomatous lesions on the ventral and lateral surface of the tail.

FIG. 77. 76 days. A large granulomatous lesion near the base of the tail with diffuse infiltration of the skin over the outer portion indicated by the glistening character of the surface.

FIG. 78. 65 days. Multiple lesions of a nodular character on the distal portion of the tail. There are beginning necrosis and ulceration of the largest of the lesions.

FIG. 79. 76 days. The same animal, showing extension of the tail involvement together with necrosis and ulceration of the affected area.

FIG. 80. 113 days. Circumscribed nodular lesions, middle and outer third of the tail. The scar of a former lesion is seen in the area marked with an arrow.

FIG. 81. 68 days. A group of three nodular lesions situated on the ventral and lateral surfaces near the base of the tail.

FIG. 82. 109 days. A diffuse infiltrative process involving the skin of the tail over its entire extent. Note the alopecia over the ventral surface of the tail and a small area of necrosis and ulceration near its distal extremity.

PLATE 77.

FIGS. 83 to 88. Periods in the history of a cutaneous lesion.

FIGS. 83 to 85. Stages of active development of the lesion. 90, 97, and 105 days.

FIG. 86. 133 days. Marked regression of the lesion.

FIG. 87. 141 days. Definite renewal of activity.

FIG. 88. 160 days. A further stage in the growth of the lesion.

(Brown and Pearce: Experimental syphilis in the rabbit. IV.)

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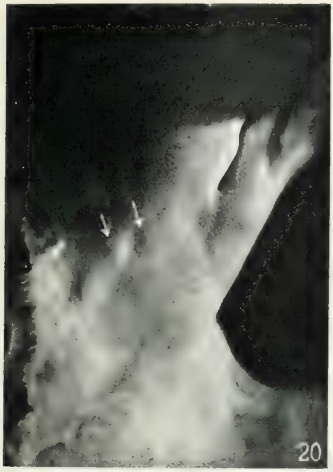
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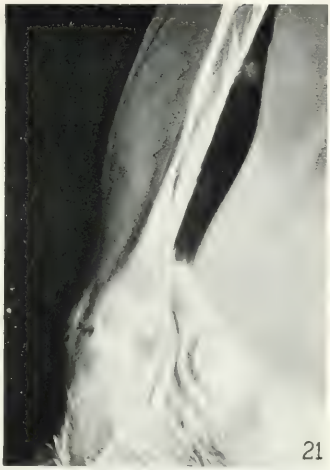
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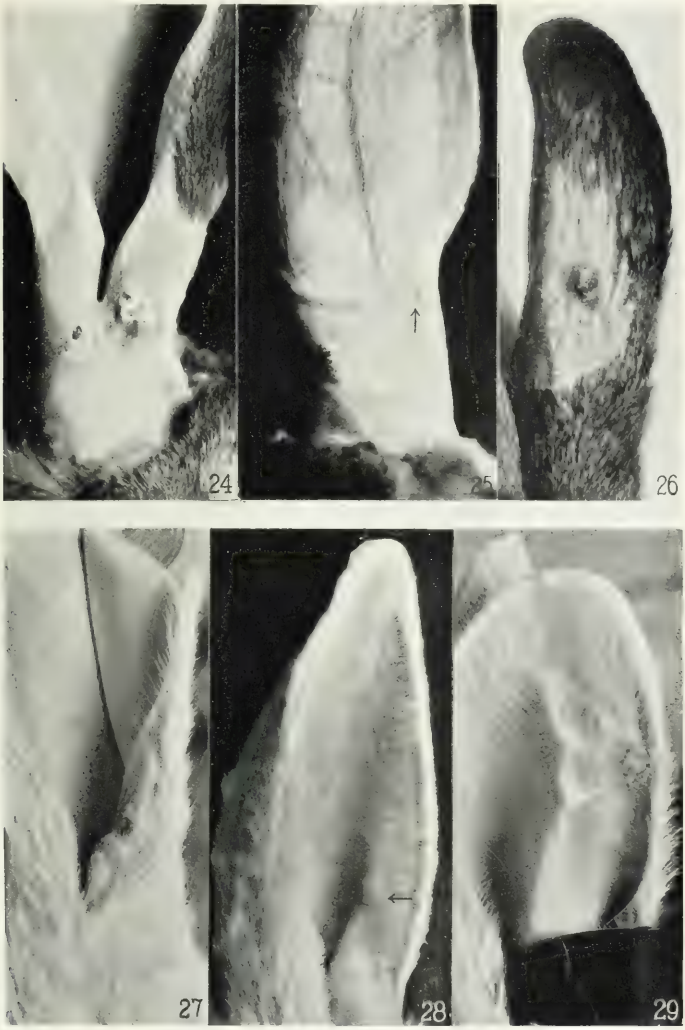
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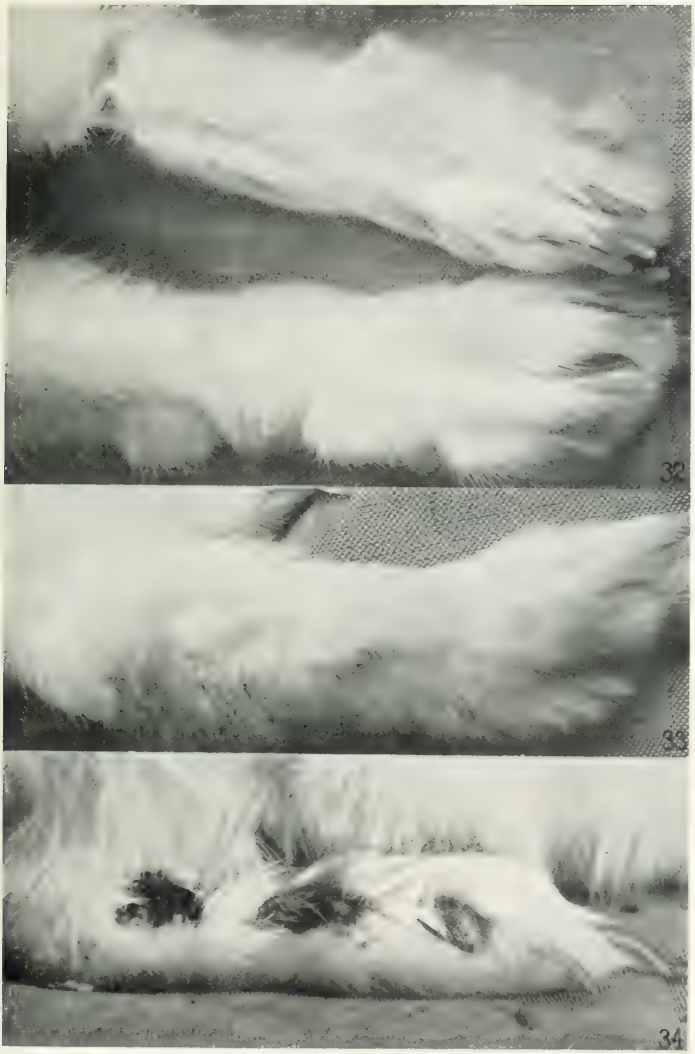

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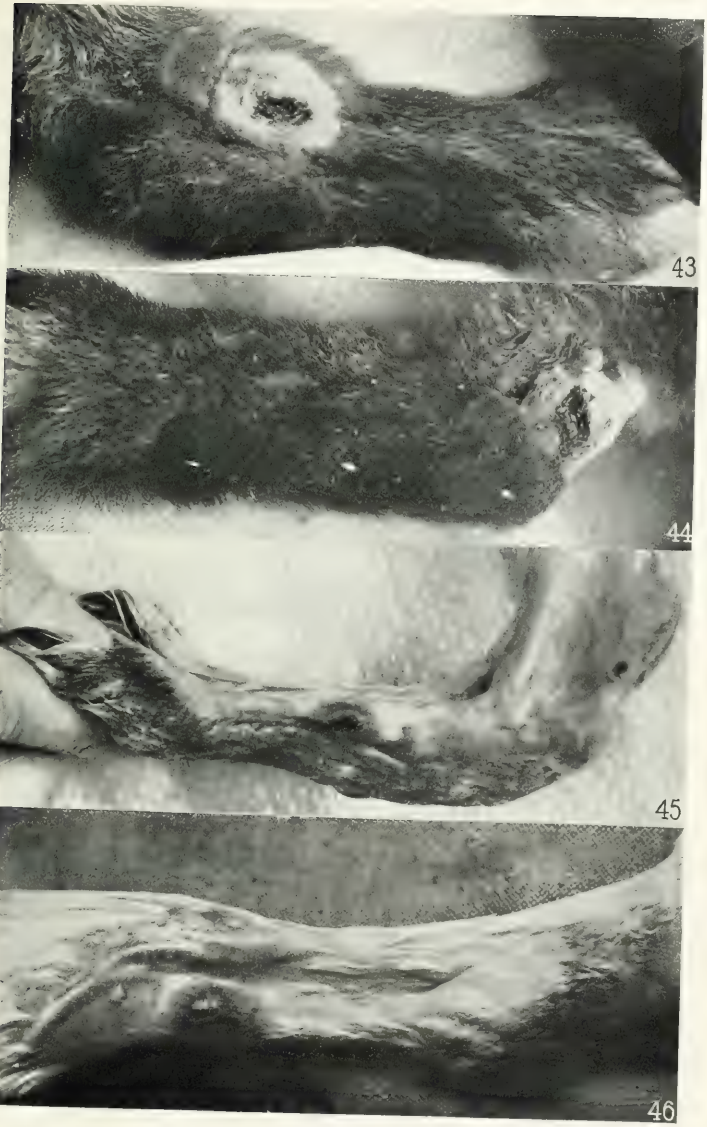

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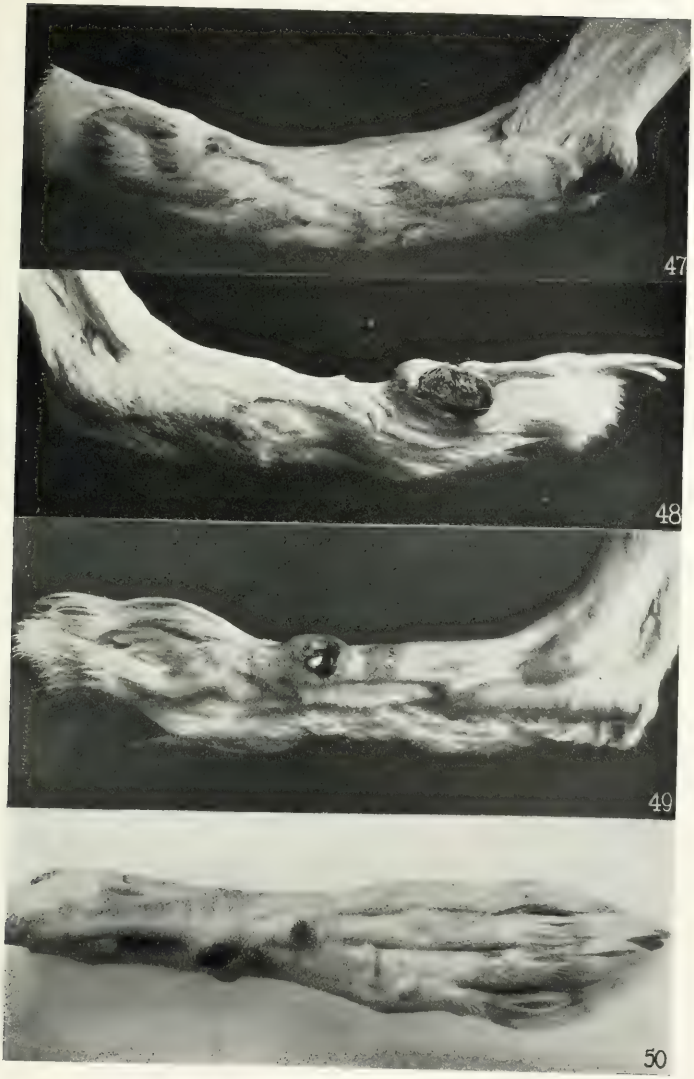

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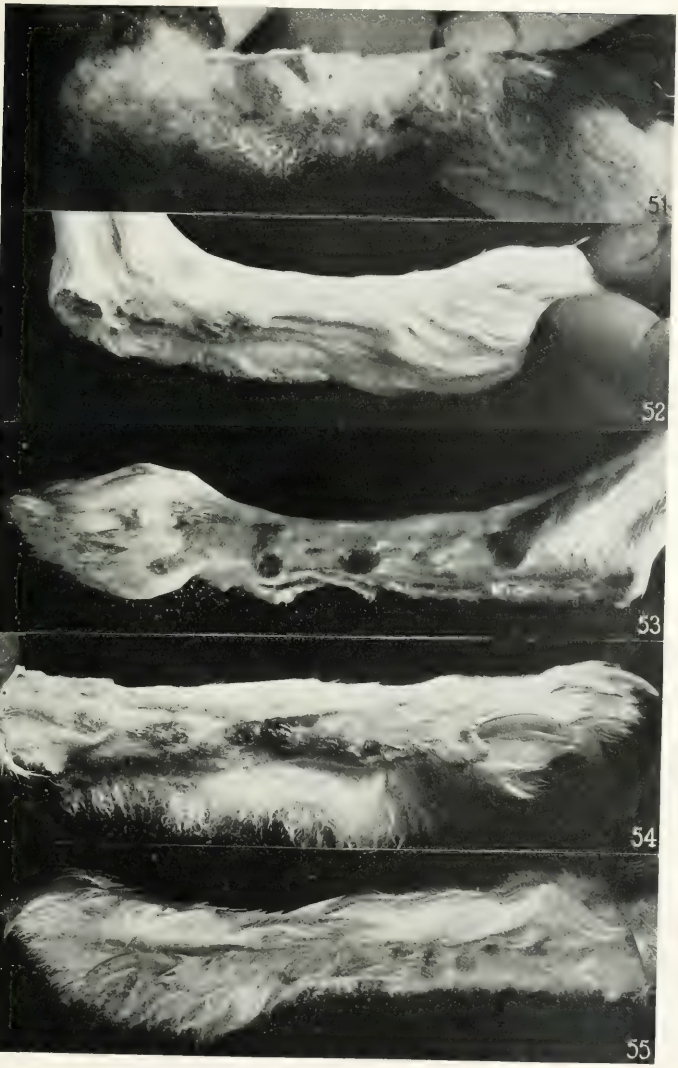

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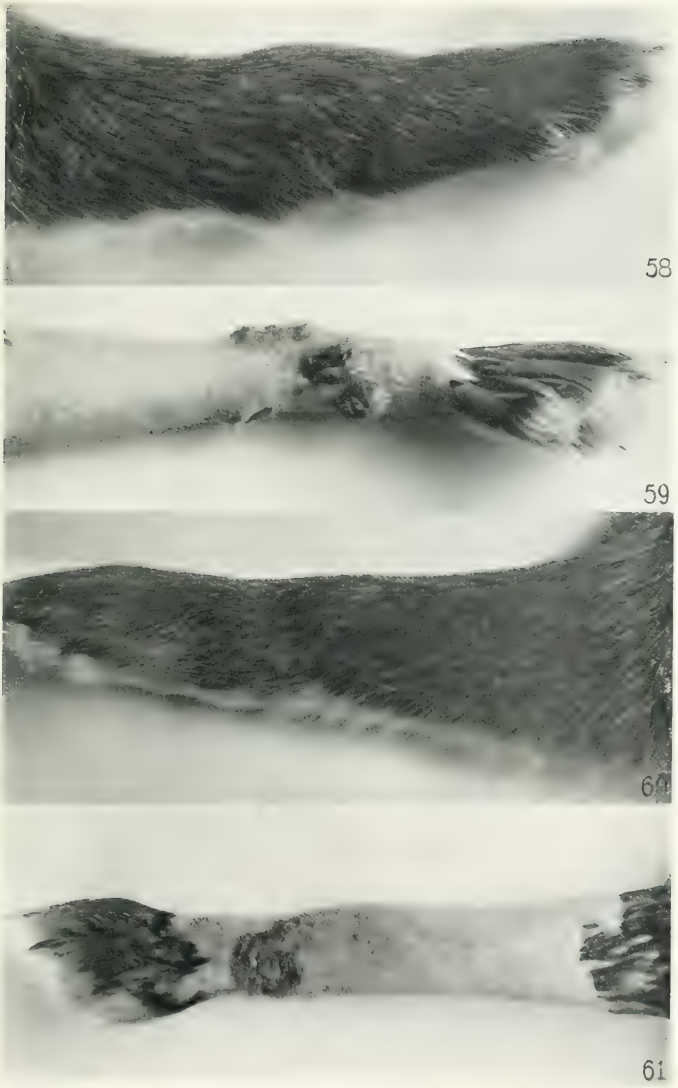
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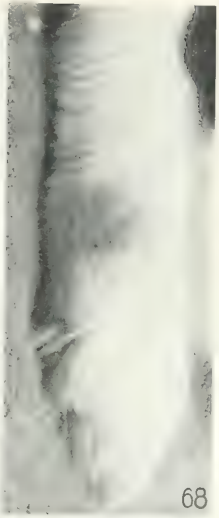

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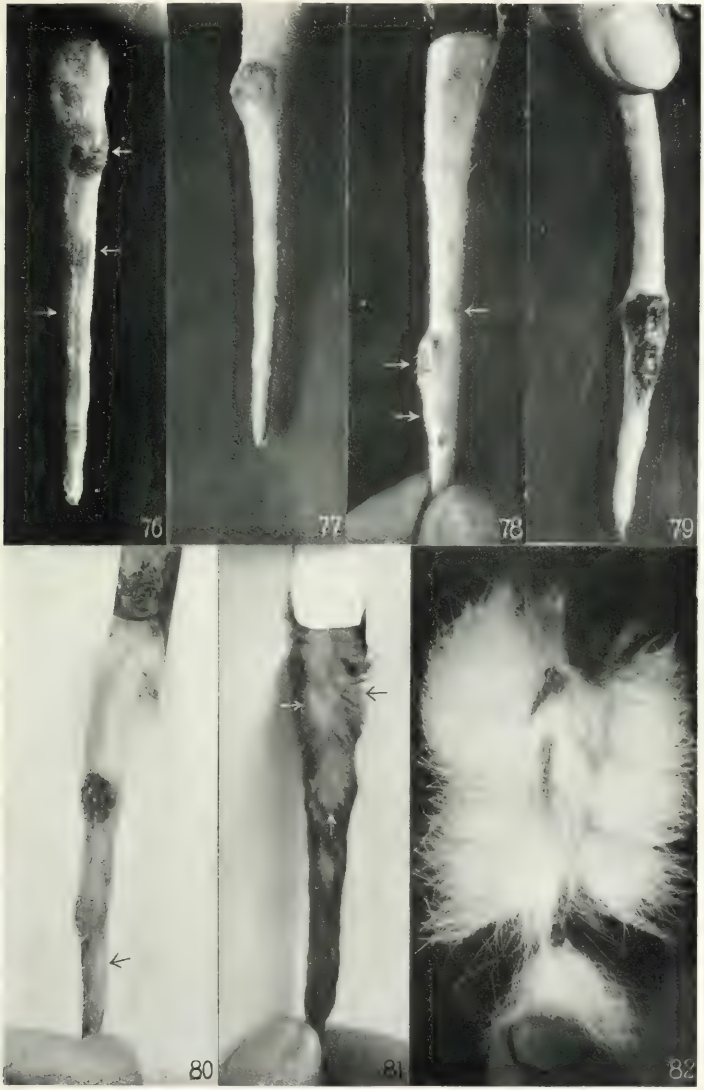
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[Reprinted from the Archives of Dermatology and Syphilology, October, 1920, Vol. ii, No. 4, pp. 470–472.]

A NOTE ON THE DISSEMINATION OF SPIROCHÆTA PALLIDA FROM THE PRIMARY FOCUS OF INFECTION.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Although it is now generally recognized that the time during which a syphilitic infection remains confined to the primary focus of entry is comparatively short, and that the effort to distinguish between a localized and generalized stage of infection is largely an arbitrary one, no one has shown just how rapidly the infecting organisms may spread after they have once gained entrance to the body or when a generalized infection may be said to have occurred. These are questions of obvious clinical importance and considerable light may be thrown on them by the sequence of events which follows local inoculations of *Spirochaeta pallida* in the rabbit.

In a recent series of experiments, an attempt was made to obtain some data on these points by a systematic determination of the presence of spirochetes in the inguinal lymph nodes and the circulating blood of rabbits at various intervals after scrotal or testicular inoculation.

The organisms used in these experiments were old strains, one of which was isolated by Nichols and Hough in 1912 and the other by Zinsser and Hopkins in 1913. They have been maintained in rabbits for periods of nearly seven and eight years.

Time of Invasion of the Regional Lymph Nodes.

The method used for determining invasion of the regional lymphatics was the excision of the inguinal nodes (under ether anesthesia) of rabbits that had been inoculated by the introduction of a small bit of infected tissue beneath the skin of the scrotum and the injection of an emulsion of these nodes into the testicles of normal rabbits. In all, inguinal nodes from twenty-nine rabbits were examined by this method.

The first series of examinations was made on rabbits showing definite primary lesions and well marked lymphadenitis in order to test the method on cases in which infection and involvement of the regional lymphatics were both apparent. The period covered by this series of examinations was from sixteen to sixty-one days after inoculation, and spirochetes were demonstrated in the nodes of all these animals.

With this data available, the time between inoculation and excision of the nodes was then progressively reduced to seven, five and two days. These periods were again fixed with reference to pathologic changes. The five and seven day periods corresponded roughly with the time at which definite enlargement and induration of the inguinal nodes could nearly always be recognized, but usually antedated the appearance of the initial lesion, while little or no alteration could be detected in the nodes within the first forty-eight hours.

There were twenty-three animals in this group. In two cases (seven days), spirochetes were demonstrated in the nodes by dark field examination and no inoculations were made from this material. The other nodes all gave positive results by animal inoculation, thus showing that extension of the infection to the inguinal lymph nodes antedated both the appearance of the initial lesion and the occurrence of definite alterations in the nodes themselves, and that this occurred constantly within a period of less than forty-eight hours from the time of inoculation.

Invasion of the Blood Stream.

Supplementing the observations on invasion of the lymphatics, a series of experiments was carried out to determine the time and frequency of blood stream invasion. The mode of determining the presence of *Spirochaeta pallida* in the blood was by bleeding from the heart, defibrinating and injecting 0.5 cc. of blood into the testicles of normal rabbits.

In the course of these experiments, a large number of bleedings was made covering the time from the appearance of the initial lesion (twelve to fourteen days after inoculation) through various periods of its development, with the result that spirochetes were shown to be

constantly present in the circulating blood by the time an infection could be recognized at the point of inoculation.

A small series of animals was then bled arbitrarily at an interval of one week after inoculation and it was found that even as early as this spirochetes were sufficiently numerous in the circulating blood to produce infection when amounts as small as 0.5 cc. were injected into the testicles of normal rabbits.

The Establishment of a Generalized Infection.

In view of the results obtained from examinations of both the inguinal lymph nodes and the circulating blood of infected animals, there seemed to be no real object in attempting to determine more exactly the time at which spirochetes could be recovered from either the lymphatics or the blood stream. However, there might be some legitimate question as to whether a dissemination of spirochetes, such as had been shown, could be regarded as a true generalized infection. In order to settle this point, ten rabbits were inoculated in the right scrotum only and forty-eight hours later the entire scrotum and testicle of this side were amputated under ether anesthesia. In spite of the complete removal of a wide zone of tissue surrounding the area of inoculation, all of these animals developed syphilitic lesions, thus showing that true infection of parts outside of the zone of operation had taken place within the brief period of forty-eight hours.

CONCLUSIONS.

While the conditions of these experiments are not entirely analogous to those that obtain in cases of human infection, the general course of events is undoubtedly much the same in the two cases.

It would appear, therefore, that, for practical purposes, there is probably no appreciable time during which a syphilitic infection can be regarded as confined to the focus of entry but that, immediately infection takes place, the spirochetes begin to multiply and invade the surrounding tissues, gaining access to both the lymphatics and the blood stream, and are widely distributed over the body even before an initial lesion can be detected.

The early appearance of a distinctive lesion at the site of infection and the lapse of time required for the development of generalized lesions are to be viewed more as a result of a sequence in localization and concentration of spirochetes at given points than as indications of the time required for their dissemination.

[Reprinted from The American Journal of Syphilis, October, 1920, Vol. iv, No. 4, pp. 593-597.]

ON THE REACTION OF PREGNANT AND LACTATING FEMALES TO INOCULATION WITH TREPONEMA PALLIDUM.

A Preliminary Note.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 12, 1920.)

The observation that a mother might give birth to a syphilitic child and suckle such a child without herself showing any obvious manifestation of syphilis formed the basis for what is known as Colles' law. By some, these circumstances were interpreted as indicating an immunity to infection on the part of the mother, but this view has been called in question and an alternative interpretation of the condition has been offered whereby the resistance of the mother would be explained upon the basis of an existing infection. This furnishes no explanation, however, for the absence of the usual manifestations of disease, if it be true that there is a certain class of women who may contract syphilis under the circumstances indicated without showing obvious signs of infection.

In some experiments carried out by us on rabbits, there was a suggestion that a clue to this anomalous situation might be obtained from a study of the reaction to infection exhibited by pregnant animals as contrasted with that of the normal. It is, of course, a well known fact that pregnant rabbits can be infected with *Treponema pallidum* and two of the most pronounced cases of generalized syphilis which have been reported were produced in this way. These animals were inoculated intravenously, however, with overwhelming doses of virus and such cases furnish little or no indication of the response which might be expected from local inoculations of the genitalia timed with reference to conception.

As an approach to this problem, two experiments were carried out with rabbits to determine the one fact of whether or not there was

any difference in the reaction of the pregnant female and that of a normal animal, whether male or female, to an ordinary local inoculation of *Treponema pallidum*, and the way in which such a difference might be expressed.

EXPERIMENTAL.

The procedure employed in these experiments was to mate normal females with males. 24 hours later, the females were inoculated on the ventral surface of the vulva near the junction of the mucous and cutaneous surfaces of the labia. The inoculations were made intradermally with 0.2 cc. of a testicular emulsion containing an average of 1 to 3 spirochetes to the microscopic field. The animals were then separately caged and held for observation. The organism used was the Nichols strain of *Treponema pallidum*.

Without entering into all the aspects of the experiments, a brief report may be given of the local and something of the general reaction exhibited by these animals during the periods of pregnancy and early lactation.

Results.

The first experiment carried out was of a preliminary character designed chiefly to give orientation. Only four females were used in this experiment and one of them proved to be fourteen days pregnant at the time of inoculation.

Two animals of this group showed absolutely no reaction at the site of inoculation. One of them has been followed for four months without the appearance of the slightest manifestation of infection either local or general. The second animal lived only 54 days after inoculation but during this time developed no local or general lesions.

The third animal of the series showed a slight infiltration along the margins of the labia which appeared between two and three weeks after inoculation. This was so slight that it would hardly have been noted had it not been known that the animal had been inoculated at this point. The infiltration disappeared completely before the birth of the young, but there was a slight recurrence which again disappeared within a short time. There was no glandular enlargement with this reaction and no other lesions have appeared during a period of more than four months' observation.

This animal gave evidence also of a constitutional disturbance in the development of emaciation and weakness. These conditions became apparent about the time the lesion appeared on the vulva and lasted for fully two months. Meantime, she gave birth to a litter of six young, one of which was born dead and four others died within a few days.

The fourth animal of the series proved to have been inoculated almost exactly at the middle of her pregnancy. There was no sign of a local reaction in this case until 54 days after inoculation or 37 days after the termination of pregnancy. The animal was then separated from her young and the lesion grew very rapidly forming a chancre of about 1 cm. in diameter.

The results obtained from these four animals were regarded as sufficiently suggestive to warrant further investigation and a second experiment was carried out in which the reaction of the pregnant animal was controlled by parallel inoculations of females in heat, normal females, and normal males inoculated on the foreskin by the same technic. There were four females mated and inoculated as in the previous experiment, two females in heat, three normal females, and three males all of which were inoculated with the same virus.

The results from the control animals may be stated very briefly. Within ten days after inoculation, the eight animals of this group all showed typical reactions at the site of inoculation together with a beginning lymphadenitis; in five of them the lesions developed very rapidly, but by the end of the third to the fourth week, all of them showed well-defined and actively growing lesions with a well-marked lymphadenitis. The vulval lesion of one animal was a diffuse infiltration about the margins of the labia and did not grow to any considerable size, but the lesions of the other animals were circumscribed, indurated chancres which reached a centimeter or more in diameter.

The reaction of the four pregnant females during the first eight weeks was quite different from that of the controls. Two of them showed no sign of a syphilitic reaction at the site of inoculation and no alterations in the inguinal nodes. A third animal showed no reaction until a few days after the birth of the young when a slight infiltration developed at the point of inoculation and disappeared within

seventy-two hours. The general condition of these three animals was unaffected.

The fourth animal of this group showed a tiny area of infiltration on the ventral surface of the vulva five days after inoculation; by the eighth day, there was a small indurated papule about 2 mm. in diameter and a slight enlargement and induration of the inguinal lymph nodes. These lesions persisted for about one week and then gradually diminished.

Meantime the animal showed a progressive weakness and emaciation; she eventually gave birth to a litter of nine young, one of which was born dead and the others lived for only a few hours. At this time, the vulval lesion was no more than a tiny copper-colored spot with no appreciable infiltration.

Four days later, a small papule appeared in the sulcus between the labia and grew to a size of about 4 mm., while the original lesion completely disappeared; there was no reaction in the lymph nodes, however, and the general condition of the animal remained unimproved at the end of the first eight weeks after inoculation.

Summarizing the immediate results of these experiments, it may be said that normal rabbits whether male or female, react to intradermal inoculations of the vulva or sheath by the prompt development of characteristic indurated lesions at the site of inoculation and a well marked lymphadenitis.

Of eight pregnant females inoculated in the same way, only four of them showed any clinical sign of infection whatsoever; in three of these, the reaction consisted of a very slight and transient infiltration at the site of inoculation, unaccompanied by lymphadenitis; one of these animals showed no constitutional disturbance, but the two in which the local reaction was most marked, showed profound constitutional disturbances as well, each giving birth to a dead fetus, and of fifteen young born of these two females, only one survived beyond the first week of life.

The only inoculation of a pregnant animal which gave rise to a lesion comparable to those of the controls was made during the middle of pregnancy, but the lesion did not appear until towards the end of lactation.

It is too early to compare ultimate effects, but the indications are that the difference in the reaction of pregnant and of normal animals as determined by other standards is fully as great as that seen in the local reaction during the first few months following inoculation.

CONCLUSIONS.

The results reported show very clearly that the reaction of the rabbit to a genital inoculation with *Treponema pallidum* which practically coincides with conception differs very decidedly from that of the normal animal inoculated in the same way and that this difference extends through the period of pregnancy and well into the period of lactation.

The differences noted are of two kinds: ordinarily, it appears that the defensive mechanism of the pregnant animal is capable of opposing a resistance to inoculations performed at the time of conception such that little or no clinical sign of infection appears—a condition possibly analogous to that upon which Colles' law was founded. In other instances, however, slight local lesions and marked constitutional disturbances are produced which suggest an ineffectual resistance to the infection or a breaking down of the defensive mechanism. From the occurrence of these two extremes, one would also expect to find a third type of condition approaching more nearly that seen in the normal animal.

The demonstration of these fundamental facts concerning the reaction of pregnant and lactating animals to inoculation with *Treponema pallidum* furnish a starting point for the investigation of a wide range of problems centering about the subjects of infection and resistance in states of pregnancy and lactation and, by contrast, may be the means of approach to the more general problem of the defensive mechanism of the normal animal.

[Reprinted from The Journal of Experimental Medicine, November 1, 1920, Vol. xxxii, No. 5, pp. 497–512.]

EXPERIMENTAL SYPHILIS IN THE RABBIT.

V. Syphilitic Affections of the Mucous Membranes and Mucocutaneous Borders.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 73 to 83.

(Received for publication, June 14, 1920.)

A study of cutaneous syphilis in the rabbit brought out the fact that although the infecting organisms might be widely distributed through the body, lesions of an easily recognizable character occurred almost exclusively within certain restricted areas while the remainder of the skin surface rarely showed any manifestation of disease (1, 2). In like manner, it has been found that lesions develop with considerable frequency in parts of the body where skin and mucous surfaces join. In some instances, the lesions first appear within the skin area, while in others they develop upon the mucous membrane so that it might be possible to classify most of them as affections of one or the other of these structures. It appears, however, that the transitional area exercises some influence upon the localization of the infection, and since lesions which develop in one tissue usually extend to the other, their classification as affections of mucocutaneous borders seems to be a more logical one.

Syphilitic involvement of the mucous membranes and mucocutaneous borders of the rabbit was first noted by Grouven (3), who described infiltrations of the nasal mucosa and rhagades about the nasal orifices, conjunctivitis, and papular lesions of the anus and sheath. Attention was also called to the presence of a nasal discharge containing spirochetes and to dyspnea presumably resulting from infiltration and swelling of the nasal mucosa. These early observations have been confirmed and amplified to some extent by subsequent observers, but no material additions have been made to the list of conditions originally described by Grouven. Uhlenhuth and Mulzer (4) described tumor-like swellings of the mucous membranes following generalized inoculations, and within recent

years, attention has been directed more particularly to the occurrence of diffuse inflammatory processes of the nose and sheath which show no characteristic lesions but are identified by a mucopurulent discharge which contains spirochetes. These observations constitute the chief contributions which have been made to the study of affections of the mucous membranes and mucocutaneous borders in the rabbit.

Among the animals first studied by us, localized infections of the mucous membranes and mucocutaneous borders were noted in about 20 per cent of the cases but have been less frequent among those studied more recently. The affections seen in these animals were of two general classes, depending upon the type of the lesions present. In one group, the lesions were characterized by diffuse infiltration, surface erosion or ulceration, and the formation of exudates of various types; in the other, there was a greater degree of proliferation and the lesions formed were large granulomatous masses which showed the usual secondary transformations of syphilitic processes. Affections of these two classes were distributed about the nares, the lips, the margins of the lids, the genitalia, and the anus.

Affections of the Nasolacrimal System and of the Nasolabial Region.

Infections of the nasolabial region were relatively infrequent, which, as will be explained later, may have been due to the fact that lesions in these areas were of comparatively late development and the animals were not held for a sufficiently long time.

The most common affection seen was a condition which in its earlier stages closely resembled an ordinary case of snuffles. The infection began as a rhinitis with a more or less profuse mucopurulent discharge and apparently extended to the nasolacrimal ducts, producing obstruction and consequent overflow of the lacrimal secretion.

At the onset of the local infection, no characteristic lesions could be detected by ordinary means of examination, but within a short time the discharge from the nose became of a more tenacious character and tended to adhere to the surfaces about the nasal orifices. There was then a noticeable reddening and swelling of the skin and mucous membranes, and removal of the accumulated discharge revealed the presence of minute abrasions as indicated in Fig. 1. An earlier stage of the process may also be seen in Fig. 9 which is a photograph of the same animal as that in Fig. 1, taken 1 month earlier.

As the local infection advanced, the skin areas became denuded of hair; the infiltration of the surrounding tissues was increased, especially along the edges of the alæ nasi, and the nares were obstructed by the presence of accumulated discharges or by thick adherent crusts, the removal of which left numerous raw and bleeding points (Figs. 1 and 2).

Several modifications of this general type of condition were seen. In some animals, the nasal discharge was comparatively slight, and the lesions were of a less diffuse character. Irregular areas of infiltration and rhagades occurred along the margins of the alæ and more especially at the angles of the nares. Occasionally also, simple areas of ulceration or ulcers surrounded by a definite zone of infiltration such as that in Fig. 3 were seen just at the edges of the nasal orifices.

Another very characteristic affection of the nasolabial region is that shown in Fig. 4. The condition as presented by this animal was a marked infiltration of the nasal mucosa and the tissues surrounding the left nasal orifice. The right side of the nose was also affected to a slight extent. The cutaneous portion of the lesion was covered by an accumulation of epithelial scales and crusts, while the mucous surface showed ulcers with a gray necrotic base.

An unusually destructive condition seen in one animal of this group is that shown in Figs. 5 to 8. The usual symptoms of acute rhinitis were absent in this case. When first detected a circumscribed, indurated mass was present in the superior portion of the right nostril and a smaller area in the left (Fig. 5). These lesions increased very rapidly, giving great breadth and prominence to the alæ (Figs. 5, 6, and 10). Depressed ulcers developed upon the mucous surfaces and spread until the skin margins were involved. Heavy reddish brown crusts were then formed and practically occluded the anterior nares on both sides (Fig. 7). The necrosis associated with these lesions was quite marked and led to considerable destruction of the soft tissues and consequent deformity of the nose (Fig. 8). At autopsy, it was found that a large part of the mucous membrane of both nasal passages had become involved and the left chamber was practically obliterated for a distance of about 2 cm. above the anterior orifice. Whether this was due to the *pallidum* infection or was the result of an associated bacterial infection could not be determined.

One other form of lesion was observed at the margins of the nares, and this also was seen in but a single animal. The lesion in this case was a discrete papule about 3 or 4 mm. in diameter situated on the lip near the median line. The tumor-like swellings of the nasal mucosa described by Uhlenhuth and Mulzer (4) were not observed in any of these animals. They have been noted by other observers (5), however, and it is possible that some of the conditions which we have described as infiltrations of the nasal mucosa may represent processes analogous to those referred to as nasal tumors.

Taken as a whole, this group of conditions presented a very characteristic appearance and resembled in many respects the nasal

affections of infants with hereditary syphilis. The essential feature of the lesions was an infiltration of the skin and mucous membranes with irregularly distributed areas of necrosis and ulceration, while the characteristic symptoms were the presence of a mucopurulent discharge, epiphora, or conjunctivitis, and dyspnea, the latter being apparently an obstructive phenomenon.

Lacrimation or lacrimal overflow has been mentioned by different writers as a symptom of infection of the nasal mucosa, and while there was a very constant association of these conditions, the causative connection between the two processes was not entirely certain. During the early stage of the nasal infection, the characteristic condition of the lacrimal system was that shown in Figs. 9 and 10. At this period, the cheeks below the anterior angle of the eye were bathed with a clear lacrimal secretion. The hair was matted together, the skin was slightly inflamed, and usually there was some loss of hair over these parts. The conjunctiva in some instances remained perfectly clear, while in others there were varying degrees of an acute inflammatory reaction. Eventually, the lacrimal secretion was altered, becoming more clouded, or mucopurulent, in character. The conjunctivæ were then dulled, and the secretion accumulated over the cheeks in greater amount as in Fig. 11.

Other workers have reported the presence of spirochetes in the lacrimal secretions, but repeated examination in these cases failed to show them. While this in itself is not conclusive, it suggests that these affections might be referable to occlusion of the nasolacrimal ducts either as a result of involvement of the nasal mucosa or of infection of the ducts themselves rather than to localized infection of the conjunctival sac. Except for its periodicity and its persistence, this feature of the nasal infection differed in no way from a similar condition frequently observed in rabbits from other causes.

Occurrence and Duration.—As has been stated, affections of the nasal region were not among the early manifestations of generalized syphilis. The majority of those seen were either late in their development or at least occurred subsequently to other types of lesions. The time at which they were first recognized varied from a minimum of 9 weeks to a maximum of 8 months after inoculation, but there were very few cases in which lesions appeared within the first 4 months.

These statements may give an erroneous impression as to the time at which the infection actually became localized in the mucous membranes of the nose or about the mucocutaneous margins, since the early

symptoms were in most cases but little more than an ordinary rhinitis and specific infection was recognized only after the condition had advanced sufficiently to arouse suspicion as to its etiology. For the same reasons, many cases of nasal infection may have passed unrecognized, since it was not possible to make routine examinations of all nasal discharges.

This group of lesions was not only late in developing but was also of a very enduring character. With a few exceptions, the animals of this group were held for a number of months after the development of the localized infection, and no instance was recorded of complete healing of the lesions during the period of observation. Two of the animals were held for approximately 1 year, and a third was under observation for more than 2 years. In the last animal, there were several periods during which the lesions about the nares underwent almost complete resolution, but each time there was a recurrence. It seems probable, therefore, that this group of conditions may be regarded as among the most persistent of the generalized infections in the rabbit.

Affections of the Lips and Buccal Cavity.

Syphilitic lesions were occasionally noted upon the mucous surfaces or along the margins of the lips, but the entire buccal cavity of the rabbit is an almost unexplored region. Small papular infiltrations were seen on the skin surfaces or at the mucocutaneous borders of both the upper and lower lips as described in the fourth paper of this series (1, 2), but even these were rare. In addition, lesions were found about the cleft in the upper lip or upon the mucous surfaces of the lips.

The region of the cleft in the upper lip was one of especial interest. Normally the contact surfaces of this area are covered by a short downy growth of hair which reaches practically to the inner margins as shown in Fig. 12. There were several animals in which an infiltration about the nasal orifices continued downward along the margins of the nasolabial folds, forming a thickened ridge, the surface of which was bare and covered by scales or by gray necrotic patches and small moist areas of erosion. A condition of this kind was present on the left lip of the animal shown in Fig. 4.

In addition to such processes as these, there were a few instances in which independent affections of the cleft were observed. The lesions appeared in the form of small papules or flattened patches of infiltration which tended to spread over the surface of the lips, and the resulting affection presented a very characteristic appearance which is shown in Fig. 13. It will be noted here that the affected portions of the lips are moist and denuded of hair, that there is a distinct thickening of the left lip, and that the surfaces are marked by an irregular network of ridges covered by gray necrotic areas. Eventually small superficial ulcers were formed which bore a striking resemblance to the mucous lesions of man.

Similar affections were also noted on the mucous surfaces of the lips, but, as a rule, these were small erosions of an indifferent character in which no spirochetes could be demonstrated. In a single instance, however, an ulcer with definitely infiltrated margins was found upon the inner surface of the upper lip (Fig. 14).

Another type of condition which was seen in a number of rabbits consisted of small papillomatous growths which were occasionally present on the margins or inner surfaces of the lips, about the gums of the lower incisors, and were especially numerous on the sides and under surface of the tongue. While these affections resembled in some respects certain of the hypertrophic or vegetating lesions of man, we could obtain no proof of a syphilitic origin—either clinical or histological.

Occurrence and Duration.—Affections of the lips were in part no more than extensions from those about the nose and were subject to the same general conditions. The independent affections of the lips appeared to be of earlier development and of comparatively short duration. There were very few of these, however, and too much cannot be inferred from such a small group of cases.

Affections of the Eyelids.

The lesions seen on the eyelids might be separated into two distinct classes according to their location, those which apparently originated in the skin and seemed to bear no particular relation to the marginal area, and those which originated at the mucocutaneous junction of the lid or ultimately involved this area. The first group of conditions was described in the paper dealing with cutaneous lesions. The affections of the mucocutaneous borders proper were also of two general types, first, papular or granulomatous lesions (Figs. 15 and 16), and second, lesions which appeared in the form of ulcers along the margins of the lids (Fig. 17).

The papular and granulomatous lesions presented no essential difference from the skin affections previously described, except as they came to involve the conjunctival surfaces. When this occurred, there was usually a moderate degree of conjunctivitis with reddening and swelling of the conjunctiva and increased lacrimation. These symptoms, however, were only transient, and within a few days, such inflammatory reactions were confined to the immediate area of the lesion (Fig. 16).

Lesions which originated upon the margins of the lids or on the surface of the conjunctiva itself usually produced a greater degree of inflammatory reaction. In most instances, they appeared, as we have said, in the form of small ulcers or abrasions. Occasionally, the initial affection was a minute papule, such as that in Fig. 15, which subsequently underwent ulceration. The appearance presented in these cases depended largely upon the extent of the induration which was associated with the formation of the ulcer. In several instances, lesions of this type developed definite collars of induration such as that shown in Fig. 17. As will be seen from this figure, there was a well marked area of infiltration extending from the margin of the lid back under the surface of the conjunctiva.

Occurrence and Duration.—In the few animals which showed lesions upon the margins of the lid, the time of appearance of these lesions varied from about 3 to 11 months after inoculation, but the majority occurred within 4 months. On the whole, they were decidedly less enduring than those about the nose, but in exceptional instances, they lasted for several months with varying periods of activity and quiescence or regression.

Affections of the Penis and Sheath.

As in the case of localized infections about the nose, it is probable that many cases of specific infection of the penis and sheath occurred which did not lead to the formation of lesions of a sufficiently definite character to attract our attention. Among the animals in this group, there were several in which the diagnosis of the local infection was made prior to the development of any characteristic lesion. These cases were suspected on account of redness and some swelling of the sheath and the presence of a mucopurulent discharge which upon examination was found to contain *Treponema pallidum*. Otherwise the diagnosis was first made on account of the presence of lesions which proved to be syphilitic.

The list of affections which occurred about the penis and sheath included first, cases of a diffuse inflammatory character associated with a mucopurulent exudate, second, circumscribed or diffuse infiltrations which usually led to the formation of papules or ulcers, and third, lesions of a granulomatous type consisting either of circumscribed masses or of more diffuse granulomatous processes.

The acute inflammatory process which marked the beginning of many of these localized infections was a fairly characteristic feature of affections of the moist surfaces or the orifice of the sheath. In one group of cases, there was a diffuse redness and swelling of the parts as in Fig. 18, which lasted for only a short time as a rule and was superseded by a diffuse infiltration (Fig. 19). As the process advanced, erosions or definite ulcers made their appearance in various localities but more especially about the margins of the sheath. Lesions on the skin surface were covered by dry, adherent crusts, while those on the mucous surfaces of the sheath and the shaft of the penis were moist and covered by a gray necrotic exudate. Occasionally small papules or condylomatous growths were also present (Fig. 18).

In another group of cases, the initial lesion was of a more circumscribed character and appeared in the form of a slightly elevated area of a gray, pink, or amber color surrounded by a zone of slight inflammatory reaction (Figs. 20 and 22). Some of these processes tended to spread and formed diffuse areas of infiltration (Figs. 20 and 21), while others developed into circumscribed and indurated nodules as was the case with the lesion shown in Figs. 22 and 23.

When the infection was confined to the skin surface, the acute inflammatory reaction was usually slight and the conditions present were much the same as in the case of other skin lesions. Thus in Fig. 24 there is seen an area of infiltration involving the right side of the sheath which is just beginning to show surface necrosis and eventually developed into the chancre-like mass seen in Fig. 25.

This type of affection was comparatively common and presented numerous variations of the condition illustrated. Two especially marked cases of infection confined to the region of the meatus of the sheath are shown in Figs. 26 and 27. It will be seen that the lesions completely surround the orifice of the sheath and are located practically at the line of junction of the skin and mucous surfaces. In one case (Fig. 26), the necrosis appeared exactly along this line and completely encircled the meatus. In the other (Fig. 27), the necrotic area occupied a similar position, but at the time the photograph was taken, the ulcer was confined to one side.

Another form of unusually marked involvement of the sheath is that shown in Figs. 28 and 29. The condition began with edema and congestion of the sheath, and, as the lesions developed, there was marked enlargement and induration of the sheath extending from meatus to base, associated with a pronounced exfoliation of surface epithelium and the formation of cracks and erosions of the skin

and mucous surfaces (Fig. 28). Eventually, an irregular line of necrosis and ulceration appeared and separated the mucous from the skin surfaces. On the whole, the mucous membrane appeared to be less involved than the skin, and no definite involvement of the penis could be made out.

Involvement of the penis, as has been mentioned, may occur along with that of the mucous surface of the sheath, in which case the lesions seen were of the type of small infiltrations or surface erosions. One other form of lesion may be mentioned which was observed in but one animal. This consisted of an elevated area of infiltration surrounding the urethral orifice as is indistinctly shown in Fig. 30. In addition, it may not be out of place to mention that infection of the mucous membrane of the urethra with the production of lesions has been demonstrated microscopically.

Occurrence and Duration.—As might be expected from their proximity to the point of inoculation, lesions of the sheath developed somewhat earlier than those of the other mucocutaneous surfaces. The earliest lesions of this group appeared 26 days after inoculation, in other words, almost as soon as the primary lesion itself. There were two other cases which occurred within 2 months after inoculation, and a number of cases within a period of 3 months, while the latest case of the series was 6 months after inoculation. The subsequent history of lesions of this group was very variable. Some lasted for a comparatively short time, while others endured for many months. For example, the lesions shown in Figs. 28 and 29 were still fairly active when the animal was killed for pathological examination $5\frac{1}{2}$ months after the lesions first appeared, and in a second animal, the lesions persisted for a little more than a year.

Affections of the Anal Region.

With a few exceptions, animals which showed specific involvement of the sheath showed lesions of the anus as well, and, conversely, there were only two animals with anal lesions in which corresponding lesions were not present on the sheath. The two groups of conditions, therefore, might almost be considered as one, and apparently the main difference between them was in the character of the lesion which occurs in the two locations, and even this was not great. There were no true exudative affections of the anus. The lesions situated on the cutaneous surfaces were essentially the same in all respects as those

of the sheath. In fact, there was a very striking similarity between the lesions present in the two localities, as may be seen by comparing those shown in Figs. 24 to 29.

The most interesting conditions seen by us in the anal region of the rabbit were lesions which might be classed as condylomata, a typical example of which is given in Fig. 31. These lesions were, as a rule, entirely obscured from observation until the anal ring was distended to a sufficient extent to permit of inspection of the transitional borders. This could be easily done by exerting pressure upon the rectum immediately behind the sphincter.

The first suggestions of the presence of lesions of this character (condylomata) came with slight redness and swelling of the anus, and during the earlier stages of the localized infection, no other alteration might be present. As infection advanced, the reaction became more localized, with the development of areas of infiltration and induration situated in one or more segments of the anal ring immediately along the junction of the skin and the mucous membrane. These were not unlike the patches of infiltration on the mucous surfaces of the sheath. In cases of more pronounced involvement, however, such as that shown in Fig. 31, the entire anal ring was involved, with extension of the process over both the mucous and cutaneous surfaces. The lesion thus formed was a distinctly elevated and indurated mass strikingly like the condyloma latum in man. Judging from our small series of animals, lesions of the condyloma type are more frequent about the anus of the rabbit than on the sheath, and this constituted the chief difference between the lesions of the two localities.

Occurrence and Duration.—As regards the time of occurrence and duration of anal lesions, they are again comparable to those of the sheath, but, on the whole, appeared to be slightly more delayed in their development and in a few instances were more enduring. The condyloma shown in Fig. 31 remained active for upwards of 15 months and still showed slight signs of activity when the animal was killed 21 months after inoculation.

Diagnosis of Mucocutaneous Infections.

Obviously there are many conditions affecting the rabbit which might be difficult to differentiate clinically from some of the affections described above. This is especially true of infections of the nose, eyes, and genitalia, but rare in the case of the anus. A syphilitic rhinitis which is not associated with characteristic lesions presents much the same train of symptoms as the more common condition

known as snuffles. The chief symptomatic difference between the two affections is in the associated involvement of the lacrimal system. As a rule, snuffles is not associated with marked and persistent lacrimal overflow, and when involvement of the lacrimal system does occur, there is usually an acute conjunctivitis. On the other hand, profuse lacrimation without an acute inflammatory reaction is very common even in the early stages of a syphilitic infection. At this stage, however, the distinction between the two processes can be made with certainty only by a demonstration of spirochetes in the nasal discharge or in the mucous membranes themselves.

In cases in which lesions are present, the differentiation of the two conditions presents less difficulty. In the most severe and long standing cases of snuffles, some infiltration and erosion may be present about the nares but they are associated with a greater degree of suppuration and could hardly be confused with the conditions described above.

The two main conditions to be considered in connection with the eyelids and conjunctiva are trauma and conjunctivitis of bacterial origin. Slight abrasions of the lids are fairly common among rabbits, especially as a result of scratching, but they usually heal very quickly and should not be confused with syphilitic infiltrations. There is also a parasitic disease of the skin (mange) which occasionally forms small lesions along the margins of the lids, but these are readily identified by the character of the lesion and the presence of a similar affection upon other parts of the body.

Traumatic conjunctivitis and epizootic infections of the conjunctiva must also be distinguished from affections of a syphilitic nature. These conditions, however, have a more immediate connection with affections of the eyes than with those of the present group.

There are several conditions, chiefly infections, which should be mentioned as possible sources of confusion with syphilitic involvement of the penis and sheath. So called gleet is a well known disease of rabbits which affects the genitalia of both males and females and is characterized by an acute inflammatory reaction with a purulent exudate and may lead to necrosis and ulceration. We have seen a few such cases among normal animals but know very little of this condition from personal observation.

Arzt and Kerl (6) have described a similar condition in rabbits due to a spirochete infection which is capable of transmission from one animal to another. It is claimed that the lesions produced by this organism bear some resemblance to syphilitic lesions. We have never encountered infections of this type.

Traumatic and pyogenic infection about the genitalia must also be considered, but as far as we are aware, the only class of syphilitic affections which might be confused with any of the conditions mentioned are those in which no characteristic lesions are present, and in these cases, a diagnosis can be made only by the demonstration of spirochetes.

SUMMARY.

In a series of more than 200 rabbits in which generalized lesions were observed following local inoculation with *Treponema pallidum*, there were a number of animals in which characteristic lesions were noted upon mucous membranes or along mucocutaneous borders. These lesions were distributed with about equal frequency between the nose or nasolacrimal system and the eyelids on the one hand, and the genital and anal regions on the other. The lips and buccal mucosa appeared to be less subject to localized infections unless the papillomatous growths noted on the lips and under side of the tongue should prove to be in some way connected with such an infection.

In many instances, the local reaction was initiated by an acute inflammatory process, and in the case of nasal and genital infections, a definite exudate was formed. The succeeding stages of the reaction consisted in an infiltration of the parts involved, together with a variable degree of proliferation of fixed tissue cells, leading eventually to necrosis and ulceration. The resulting lesions differed according to their location and the character of the reaction in the individual case. Localized infections of the nose occurred in several forms, first, as a rather diffuse affection of the nasal mucosa characterized by the presence of a mucopurulent exudate, second, as a more or less circumscribed process of infiltration with an especial predilection for the region of the anterior nares, and third, as a granulomatous process involving the alæ in particular.

Involvement of the nasal mucosa was very commonly associated with lacrimal overflow and with some degree of conjunctivitis.

The lesions of the eyelids were usually small, elevated papules or lesions of an ulcerative character some of which were surrounded by a zone of infiltration. In exceptional instances, large granulomatous lesions occurred along the margins of the lower lids.

Infection of the penis and sheath gave rise to conditions analogous to those of the nose. In one group of animals, there was a diffuse affection characterized by redness and swelling of the parts with a mucopurulent exudate, in another there were circumscribed or diffuse infiltrations, while in a third the lesions formed were indurated granulomatous masses. Secondary necrosis with erosion or ulceration was a common feature of all these conditions.

Localized infections in the region of the anus differed from those in other localities chiefly in the absence of an exudative group of affections and in the frequency with which lesions of a papillomatous type occurred.

Lesions of mucous membranes and mucocutaneous borders developed at periods of time varying from a few weeks to several months after inoculation. Most of them were rather enduring and in several instances persisted in an active condition for considerably more than a year.

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EXPLANATION OF PLATES.

The illustrations are reproductions of unretouched photographs which, with the exception of Fig. 3, represent the objects at their natural size. Fig. 3 is at a magnification of 1.5. Statements of time are estimated from the date of inoculation unless otherwise indicated.

PLATE 78.

FIGS. 1 to 4. Syphilitic affections of the nasolabial region.

FIG. 1. 4 months. Syphilitic rhinitis with infiltration about the nares. There is loss of hair over the affected area, with superficial necrosis and ulceration and the formation of adherent crusts.

FIG. 2. 10 months. Marked infiltration of the alæ with necrosis and the formation of ulcers covered by crusts. There were also pronounced lesions at the angles of the nares.

FIG. 3. 68 days. A circumscribed and elevated area of infiltration with central necrosis and ulceration situated in the right nasal orifice. The lip is drawn down to bring the lesion into view.

FIG. 4. 5½ months. A marked diffuse infiltration of the skin and mucous membrane of the left side of the nose and the nasolabial fold with slight involvement on the right. The skin area shows exfoliation of surface epithelium and there were erosions on the mucous membrane.

PLATE 79.

FIGS. 5 to 8. Stages in the progress of an ulcerating granulomatous lesion of the nose.

FIG. 5. 4½ months. Early granulomatous lesions in the anterior nares with an ulcer on the right. There was no nasal discharge at this time.

FIG. 6. 2 weeks later. A slightly later stage of the affection showing the marked prominence of the alæ and the extension of the necrosis and ulceration, now present on both sides.

FIG. 7. 7 months after the appearance of the lesions. There has been marked destruction of the soft tissues of the nose, and both nostrils are occluded by thick reddish brown crusts.

FIG. 8. The same lesions 1 week later with the crusts removed. There was still slight activity in some parts of the lesions most noticeable on the right. The left nasal orifice was entirely obliterated, and there was only a small opening on the right.

PLATE 80.

FIGS. 9 to 11. The clinical appearance presented by animals showing a combined nasolacrimal involvement.

FIG. 9. 3 months. The same animal as that in Fig. 1, showing an earlier stage of the affection. Note the characteristic mucopurulent discharge about the nose associated with lacrimal overflow. This condition is not unlike that sometimes seen in snuffles.

FIG. 10. 5 months. The same animal as that in Figs. 5 to 8. A pronounced lacrimal overflow was present in this animal without an associated nasal discharge. Note the swollen condition of the lids and skin at the anterior or internal angle of the eye.

FIG. 11. The same animal at a later period of the infection (1 year after inoculation). Chronic dacryocystitis.

PLATE 81.

FIGS. 12 to 14. Affections of the labial cleft.

FIG. 12. The appearance of the surface of the labial cleft in a normal rabbit.

FIG. 13. 3 months. A diffuse infiltration of the skin and adjacent mucous surfaces of the labial cleft, more marked on the left than on the right. The skin is denuded of hair on both sides, and the left lip shows a series of irregular ridges covered by gray necrotic epithelium. Mucous patches.

FIG. 14. 3½ months. A sharply circumscribed and indurated ulcer on the inner surface of the left upper lip.

FIGS. 15 to 17. Lesions of the margins of the lids.

FIG. 15. 2½ months. An early papular lesion arising in the margin of the lower lid.

FIG. 16. 3 months. The lower lid everted to show the inflammatory reaction on the conjunctival surface resulting from a syphilitic lesion on the lid.

FIG. 17. 6 months. An indurated ulcer on the upper lid and congestion of the conjunctival vessels.

PLATE 82.

FIGS. 18 to 25. Affections of the penis, sheath, and anus.

FIG. 18. 4½ months. Diffuse infiltration of the sheath with edema and congestion of both the penis and sheath. There are small papular lesions on the sheath and an area of erosion at the superior margin of the fold in the sheath.

FIG. 19. 145 days. A later stage of the lesions in Fig. 18 showing diffuse infiltration and thickening of the sheath.

FIG. 20. 47 days. A small circumscribed area of infiltration on the mucous surface of the sheath with a diffuse redness and swelling. Sheath retracted.

FIG. 21. 2 weeks later. The same animal. The infiltration has become more pronounced and retraction of the sheath is difficult. Congestion and edema have subsided.

FIG. 22. 2½ months. An early papule situated in the transitional area between the skin and mucous surfaces of the sheath. The papule is surrounded by a slight zone of acute inflammatory reaction.

FIG. 23. 2 weeks later. The same animal. The lesion on the sheath has developed into an indurated nodule with central necrosis and ulceration. The edges of the ulcer are inverted and there are marked vascularization and redness of the tissues at its base (mucous surface).

FIG. 24. 3 months. An irregular area of infiltration in the skin of the sheath. The surface of the lesion shows beginning necrosis at two points. On the dorsal surface of the anus, there is also an indurated granulomatous lesion with a depressed ulcer at the center.

FIG. 25. 1 week later. The same animal. Both lesions have increased somewhat and are now of essentially the same character.

PLATE 83.

FIGS. 26 to 31. Affections of the penis, sheath, and anus.

FIG. 26. 3 months. Syphilitic lesions of the anus and sheath situated at the mucocutaneous borders and completely encircling these parts. The lesions were characterized by an intense induration and the development of a line of necrosis which practically coincided with the transitional area.

FIG. 27. 3 months. A similar group of lesions in another animal. The lesion on the anus, however, was confined to one side.

FIG. 28. 3 months. A diffuse infiltrative process involving the entire sheath and anal ring. The surface of the lesion was marked by irregular areas of superficial necrosis and exfoliation; there were also erosions on the mucous membranes.

FIG. 29. 1 month later. The same animal. Note the appearance of a line of necrosis separating the swollen and everted mucous membrane from the skin of the sheath. There was a similar condition of the anal ring.

FIG. 30. 2 months. A small mass is seen on the under surface of the penis (marked by an arrow), which was formed by a zone of infiltration surrounding the meatus of the urethra.

FIG. 31. 6 months. Condyloma latum of the anus The anus is everted.

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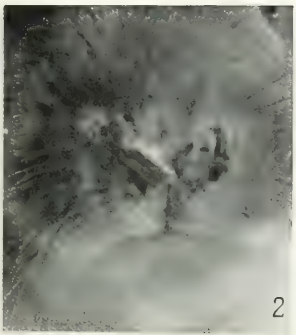
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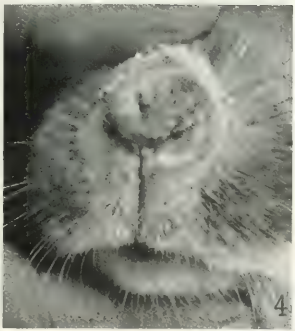
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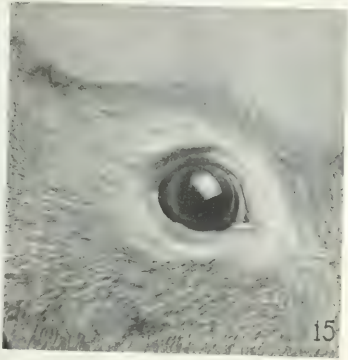
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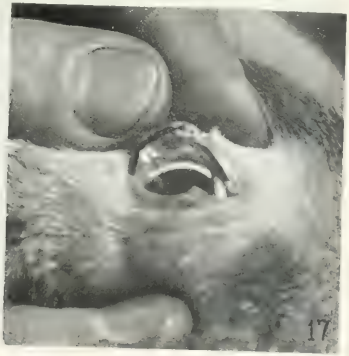
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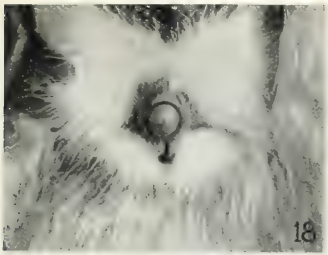
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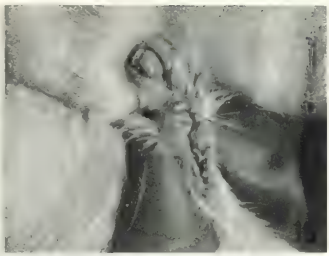

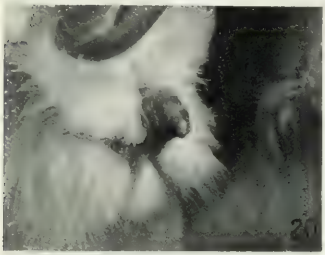
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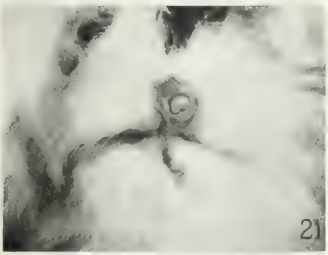
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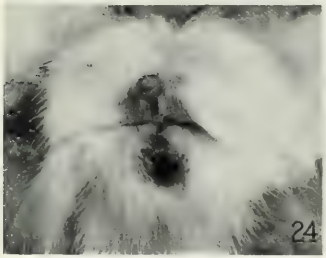
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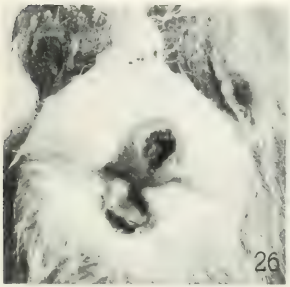
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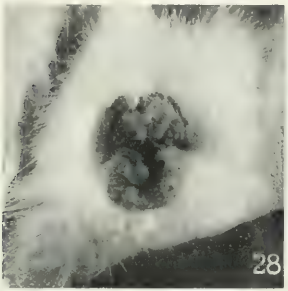
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EXPERIMENTAL STUDIES ON YELLOW FEVER OCCURRING IN MERIDA, YUCATAN.

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INTRODUCTION.

In 1918 a leptospira was detected in the blood and liver from certain cases of yellow fever in Guayaquil.[1] The organism was demonstrated also in the blood and in organ emulsions from guinea pigs which had been inoculated with blood or liver emulsions from yellow fever patients and had shown typical symptoms of the disease, fever, hemorrhages, jaundice, and nephritis. A pure culture of the same organism was made directly from the blood of yellow fever cases and the blood of experimentally infected guinea pigs, and the pathogenicity of the culture was demonstrated in guinea pigs, marmosets, and pups. The organism, which has been designated *Leptospira icteroides*, was found to be a filter passer, easily destroyed by a temperature of 55°C. within 5 minutes, an obligatory aerobe, and requiring for growth a certain amount of unmodified blood serum from man or suitable animals. Its growth is usually suppressed by secondary bacterial contamination, but when successfully obtained it remains almost invisible to the naked eye, forming neither a discrete colony nor a striking change of the culture medium, and hence the culture may be overlooked under ordinary circumstances as sterile. An ordinary microscope does not render it visible in the fresh state, and staining with ordinary dyes fails to bring out its presence. Through a dark-field microscope with a powerful illumination, however, it can readily be recognized. In the blood of yellow fever patients the organism may be present in such small numbers that an average dark-field search usually misses it. From the comparatively small percentage of positive transmissions from yellow fever patients to guinea pigs, it seems probable that many strains of the organism fail to infect this animal fatally or even with moderate severity. Whenever a secondary bacterial infection occurs after an attempt at transmission, either the animal dies before the infection with *Leptospira icteroides* fully develops, or the course of infection is atypically modified and the strain thereby lost. The various factors just enumerated no doubt constitute some of the reasons why animal inoculation or the detection of the organism has not been successful in the hands of different investigators at an earlier period.

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585.

It has been shown also[2] that the majority of the serums from persons recently recovered from an attack of yellow fever possess a specific dissolving power upon the organism when tested by the Pfeiffer reaction. This phenomenon has been regarded as significant in establishing a possible relation between yellow fever and *Leptospira icteroides*. Another important point with respect to the etiological relation was furnished by the experimental transmission of the leptospira infection from yellow fever patients to normal guinea pigs, or from infected guinea pigs to normal guinea pigs, by the bite of infected stegomyia females,[3] or with an emulsion of such mosquitoes, 8 days or longer after they had fed on infected individuals or animals.

By analogy *Leptospira icteroides* presented the principal characteristics of the virus of yellow fever as experimentally determined by Reed, Carroll, Agramonte, and Lazear[4] and by later investigators (Marchoux, Salimbeni, and Simond,[5] and Parker, Beyer, and Pothier[6]). Both are filter passers, readily killed at 55°C., transmitted by *Stegomyia calopus* after a period of incubation, not amenable to cultivation in ordinary media, invisible under the ordinary microscope, not resisting bacterial putrefaction, but capable of preservation in citrated blood under a layer of liquid paraffin for several days at room temperature,[5] inoculable by hypodermic inoculation (in the case of the yellow fever virus inoculation was made into volunteer human beings,[4, 5] in the case of *Leptospira icteroides* into susceptible animals), and producing similar symptoms. From these circumstances it seemed certain that *Leptospira icteroides* was responsible for the infection in the cases from which the organism was isolated or in which a positive Pfeiffer reaction was obtained, but the question as to whether or not *Leptospira icteroides* is also responsible for the disease known as yellow fever elsewhere than in Guayaquil was left open to further experimental determination.

The present paper gives the results obtained by us during a recent expedition to Merida, Mexico.[7] Merida, a city of 100,000 inhabitants, and the capital of Yucatan, has a small proportion of non-

[2] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.

[4] Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., *Senate Doc. No. 822, 61st Cong., 3rd Sess.*, 1911, 56.

[5] Marchoux, Salimbeni, and Simond, *Ann. Inst. Pasteur*, 1903, xvii, 665.

[6] Parker, H. B., Beyer, G. E., and Pothier, O. L., *Bull. Hyg. Lab., U. S. P. H., No. 13*, 1903.

[7] This expedition was undertaken under the auspices of the International Health Board of The Rockefeller Foundation and The Rockefeller Institute for Medical Research during the months of Dec., 1919, and Jan., 1920. The Commission received hearty and efficient cooperation from the hospital and sanitary officials in Merida, to whom we wish to express our appreciation and thanks.

immune foreign population, but it owes its previous epidemics of yellow fever to the occasional influx of foreigners or of troops sent thither from other and mountainous states where there is no yellow fever. It is reported that a systematic anti-stegomyia campaign had kept the city free from yellow fever during the 4 years preceding 1919, and that it was owing to an enormous crop of stegomyias in 1919 that an epidemic broke out in July and lasted until November, claiming about 100 patients among the newly arrived troops. The death rate was about 50 per cent.

At the time when one of us arrived in Merida,[8] there had been no new case of yellow fever for 13 days. On December 15 a soldier, J. M., was brought to the yellow fever hospital in a moribund condition and died the following morning. Postmortem examination, performed 4 hours after death by Dr. A. Lara, Director of the Hospital, and Dr. Diego Hernandez, a representative of the Department of Public Health and Charities of Mexico, established the diagnosis of yellow fever. Blood from the heart and pieces of liver and kidney were obtained for experimental purposes. The details of the experiments will be given later under the heading Results obtained with Case 1.

As soon as the diagnosis of yellow fever had been established in Case 1, Dr. Hernandez ordered the soldiers (eleven in all) who had been quartered with this patient to the yellow fever hospital, there to be placed under quarantine. Among these eleven was a young soldier, A., who was apparently convalescing from a recent attack of yellow fever. The urine and serum of this individual were studied later and will be referred to in the report as Case 3.

On December 18 one of the exposed soldiers, M., began to complain of an illness which suggested strongly the beginning of yellow fever. A systematic study of this case was started at once, the blood being withdrawn from the median basilic vein at first daily and then every other day (December 18, 19, 20, 22, and 24) and used on the one hand for cultivation and on the other, if possible, for inoculation into guinea pigs. As Chart 7 shows, the fever became typical within 3 days, with the development gradually of all other symptoms, congested conjunctivæ, flushed face, muscular pains in the limbs and loins,

[8] Dr. Kligler arrived Dec. 12, 1919, Dr. Noguchi Dec. 23.

severe headache, swollen gums, nausea, epigastralgia, albuminuria, and cylindruria. On December 24 there occurred the characteristic "coffee-grounds" vomit, also definite jaundice, which became much deeper within the next several days. The albumin and casts gradually increased in the urine, and there was an abundance of bile pigment, the amount of urine being also diminished. The patient was discharged at the end of 3 weeks. Before discharge the serum and urine were collected for further study. This case will be referred to as Case 2.

RESULTS OBTAINED WITH CASE 1.

Case 1.—J. M., soldier; brought to the Casa de Salud (the yellow fever hospital) from the barracks in the night of Dec. 15, 1919, in a critical condition, with characteristic black vomit and jaundice. Death occurred at 6 a.m. and, autopsy was made at 10 a.m., 4 hours post mortem.

Autopsy.—There was general jaundice, which was particularly marked in the scleras. The face was covered with black vomit. The liver was dotted with small yellowish areas. There were petechial hemorrhages in the stomach wall; the kidneys were hemorrhagic; the pericardium contained yellowish fluid; the serum was icteric.

Blood was drawn from the heart, and a portion of the liver and kidney was removed at autopsy for the experiments to be described. For the purpose of transmission guinea pigs were used throughout the present work.

Transmission Experiments with the Blood, Liver, and Kidney of Case 1.

Blood.

On December 16, 1919, intraperitoneal inoculation of 1 cc. of the heart's blood[9] was made into two guinea pigs (Nos. 1 and 2). Both had a temporary rise of temperature after 7 days but returned to normal within a few days. No jaundice developed at any period. On examination after 18 days there were some suspicious lesions in the lungs, otherwise the findings were negative.

[9] The same specimen of blood kept at 8°C. for 8 days was inoculated into Guinea Pigs 18 and 35 intracardially, but neither symptoms nor high temperature were seen to develop within 2 weeks (negative).

Kidney.

An emulsion of the kidney in sterile saline solution was injected on December 16 into two guinea pigs, one of which showed a very suggestive reaction (Guinea Pig 3).

Guinea Pig 3 (*Chart 1*).—Dec. 16, 1919. Intraperitoneal inoculation of 1 cc. of the kidney emulsion. Temperature rose to 103° F. in the afternoon of Dec. 19 and was 103° a.m. and 104.6° p.m. on Dec. 20. The animal was killed for transfers to three new guinea pigs on Dec. 21.

Transfers from Guinea Pig 3 (*Second Generation*).—December 21. 1 cc. of an emulsion prepared from the liver and kidney from Guinea Pig 3 was inoculated intraperitoneally into each of two normal guinea pigs, and 1 cc. of the heart's blood into a third. One died early of a secondary infection, and another remained well after having shown a slight elevation of temperature, while the third animal (Guinea Pig 3c) had definite signs of the leptospira infection.

Guinea Pig 3c (*Chart 2*).—Inoculation of 1 cc. of the blood of Guinea Pig 3. This animal began to show fever (103.2°) 5 days after the inoculation. The following morning the temperature remained at 103.2° and in the afternoon reached 105.6°. The animal was killed for examination and transfer.

Autopsy.—There was a light yellowish color throughout the subcutaneous tissues. The lungs showed numerous hemorrhagic foci, the gastric mucosa was highly congested, but there were no macroscopic hemorrhages in the latter. The intestines were icteric and congested, the liver was slightly icteric, the kidneys and adrenals were moderately congested, and the spleen was normal. The urine was slightly turbid and contained a considerable amount of albumin (++) and a few casts.

The blood from the heart of Guinea Pig 3c, as well as emulsions of the liver and kidney, were used to inoculate nine new guinea pigs. Two of the three inoculated with the blood and three of the six inoculated with the mixture of emulsions of the liver and kidney succumbed to a secondary infection with the paratyphoid bacillus. Two, however, of those escaping the secondary infection showed temperature curves and lesions quite typical of *Leptospira icteroides* infection, as described below.

Transfers from Guinea Pig 3c (Third Generation).—

Guinea Pig 54 (Chart 3).—Dec. 27, 1919. Received 1 cc. of the heart's blood from Guinea Pig 3c intraperitoneally. The temperature rose on the 7th day, and the animal remained febrile for 48 hours. It was killed for transfer and examination 10 days after the inoculation.

Autopsy.—The lungs showed numerous hemorrhagic foci. The gastrointestinal mucosa was highly congested. The liver was pale, perhaps slightly yellowish, with granular surface and several hemorrhagic spots. The kidneys were apparently swollen and degenerated, the adrenals were small and hemorrhagic, and the spleen was swollen.

Guinea Pig 59 (Chart 4).—Dec. 27, 1919. Received 1 cc. of a mixture of the liver and kidney emulsion from Guinea Pig 3c intraperitoneally. The temperature rose to 104° F. on the 6th day, and was 103° a.m. and 105° p.m. the following day. The animal was killed for transfer and examination on that day.

Autopsy.—There was a slight yellowness throughout the body, especially on the abdominal wall; no subcutaneous or intramuscular hemorrhages. The lungs showed several petechial hemorrhages on the posterior surface; there was a general congestion of the lobes. The liver was slightly icteric. The gastrointestinal tract showed scattered hemorrhagic areas, especially in the mucosa of the large intestine. The kidneys were highly congested, the spleen was normal, and the urine icteric.

Guinea Pigs 54 and 59 undoubtedly represented a positive transmission. The heart's blood and emulsions of the kidney and liver of Guinea Pig 59 were inoculated intraperitoneally into six normal guinea pigs, but the animals were lost through secondary bacillary infection.

Liver.

A small piece of the liver from Case 1 was emulsified in a sterile mortar with saline solution, and 1 cc. of the emulsion was inoculated into each of two guinea pigs (Nos. 5 and 6) intraperitoneally on December 16, 1919. One of them (Guinea Pig 6) presented a definite picture of the leptospira infection, while the other gave a suggestive reaction only. The protocol of Guinea Pig 6 and details regarding further transfers are given below.

Guinea Pig 6 (Chart 5).—Dec. 16, 1919. Received 1 cc. of the liver emulsion intraperitoneally. As the chart shows the temperature rose sharply (103° F.) in the afternoon of the 7th day and still more the next day (104° a.m. and 104.9° p.m.). On the 9th day it fell to 100.5° with the simultaneous appearance of a

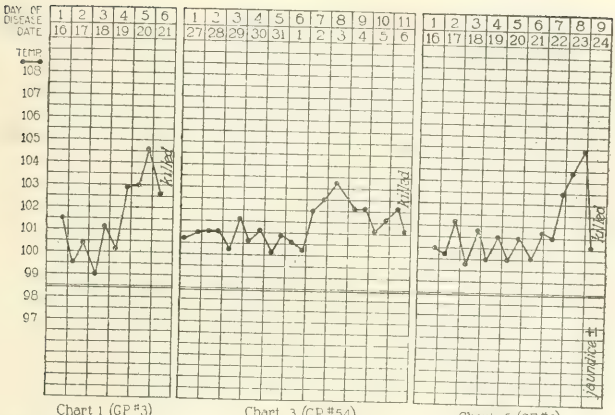


Chart 1 (GP#3) Inoc. with human kidney (Case1).

Chart 3 (GP#54) Transfer from GP#3c.

Chart 5 (GP#6). Inoc with human liver (Case1).

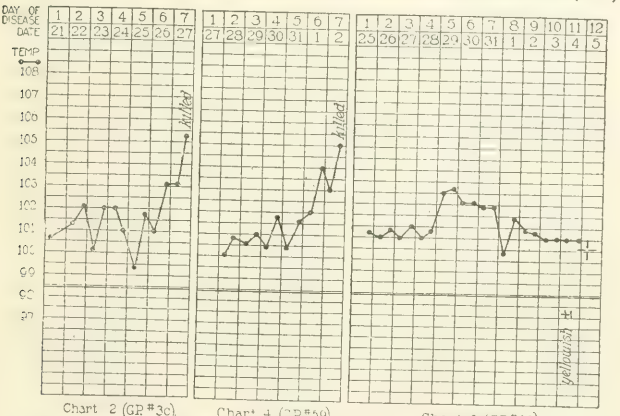


Chart 2 (GP#3c). Transfer from GP#3.

Chart 4 (GP#59) Transfer from GP#3c

Chart 6 (GP#33) Inoculated with culture from Case1

CHARTS 1 to 6. Transmission experiments with autopsy material from Case 1.

trace of jaundice in the scleras and abdominal wall. The animal was killed for transfer and examination.

Autopsy.—Faint jaundice throughout the body. Lungs showed a few ecchymoses and infarctions. Liver congested, with numerous subcapsular hemorrhagic mottles, perhaps more brownish than normally. Stomach and intestines highly congested. Kidneys and adrenals highly congested, the former showing a parenchymatous degeneration. Spleen normal. Urine turbid, brownish, containing casts and albumin.

Guinea Pig 6 represented a positive transmission, and from this animal transfer was made to three normal guinea pigs (Nos. 19, 27, and 28). The results obtained in these three animals were, first, a definite febrile reaction on the 4th day, followed by an irregular fluctuation of temperature during the several successive days (indicative of a secondary infection) without jaundice at any time. These animals were killed on the 13th day for examination. The lungs showed more or less numerous hemorrhagic foci; the other organs appeared normal. No further passage was made with this material.

Transmission Experiments by Means of Cultures from the Blood of Case 1.

Attempts were made to obtain a culture of *Leptospira icteroides* with the blood obtained from Case 1. Into each of a series of culture tubes containing the medium previously found suitable[10] for the growth of *Leptospira icteroides* 0.1 to 0.5 cc. of the blood was introduced, and the tubes were placed in a thermostat, the temperature being about 26°C. A portion of blood was also mixed with sodium citrate saline solution (equal parts) and after being kept at room temperature (26°C.) for 24 hours was inoculated in amounts of 0.1 to 0.5 cc. into three tubes on December 17, 1919.

Nearly all these tubes were found to be contaminated with a *coli*-like bacillus, owing undoubtedly to a terminal or postmortem invasion by this organism of the blood circulation. On December 25 two culture tubes, inoculated on December 17, appeared to be free from any secondary bacterial contamination. Under the dark-field microscope no leptospira could be found. From each of these two tubes about 1.5 cc. of the uppermost layer of the medium, just beneath

[10] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

the paraffin oil, were taken out by means of small sterile pipettes and the mixture of the two cultures was used for inoculating three guinea pigs. Two of the animals died of a secondary infection within 2 days, and the third (Guinea Pig 33) was found dead on the 12th day after having had slight fever from the 5th to 7th day. The protocol of this animal is given below.

Guinea Pig 33 (*Chart 6*).—Dec. 25, 1919. Inoculated intraperitoneally with 1 cc. of the mixture of Culture Tubes 1 and 2 which had been kept 8 days at room temperature after inoculation with citrated blood from the heart of Case 1 at autopsy. The temperature rose to 103° F. a.m. and 103.2° p.m. on the 5th day, and was 102.6° and 102.4° respectively for the 2 following days. It returned to normal on the 10th day, and death occurred during the night of Jan. 4, 1920, 11 days from the time of inoculation.

Autopsy.—The cadaver had undergone considerable postmortem decomposition, owing to the warm temperature of the laboratory, when found lying dead in the cage on Jan. 5. Notwithstanding postmortem discoloration, there was a slight but distinct yellowness in the skin and scleras. On opening the body also the postmortem changes greatly interfered with the efforts to determine any characteristic lesions. The lungs were congested and showed a few old hemorrhagic areas. There was a small localized abscess on the abdominal wall, and the peritoneal cavity contained some turbid fluid.

It is difficult to determine whether or not the temperature curve and lesions in Guinea Pig 33 were due wholly to the abscess or in part to a mild leptospira infection.

RESULTS OBTAINED WITH CASE 2.

Case 2 (*Chart 7*).—P. M., soldier; exposed to infection through the preceding case. Dec. 17, 1919, 5 p.m. Complained of headache. Dec. 18. Increase in general malaise; headache; rachialgia; pains in the limbs. Blood was drawn at 4 p.m. Temperature 100.9°; pulse 96. No albumin in urine. Dec. 19. Temperature 101.5°; pulse 92. Blood drawn in the morning; citrated and inoculated into two guinea pigs. Trace of albumin. Dec. 20. Temperature 103.5°. Blood drawn and inoculated into three guinea pigs. Albumin 0.05 gm. Dec. 21. Trace of albumin. Dec. 22, 3 p.m. Temperature 99.3°; pulse 78. Conjunctiva injected; scleras slightly icteric. Vomited. Stool chocolate color. Urine turbid; albumin 0.18 gm. Blood drawn for culture. Dec. 23. Albumin 1.4 gm. Pulse 72. Dec. 24. Albumin 1.8 gm.; casts and renal epithelium. Jaundice marked. Blood drawn for culture and animal inoculations. Pulse 72. Dec. 25. Albumin 2.2 gm. Dec. 26. Albumin 5.5 gm. Dec. 27. Albumin 0.8 gm.

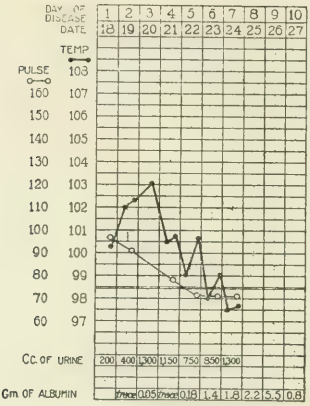


Chart 7 (patient).
Temperature curve, Case 2.

Transmission Experiments with Blood of Case 2.

First Specimen of Blood, Drawn on the 2nd Day of the Disease, December 19, 1919.

1 and 2 cc. respectively of the citrated blood were inoculated intraperitoneally into two guinea pigs (Nos. 7 and 8).

Guinea Pig 7.—The animal had a temperature of 103° F. on the 5th day; during the following 48 hours it was normal but rose again to 102.8–102.5° for 3 succeeding days. On the 12th day it was normal. On the skin of the abdominal wall were several ecchymotic areas. The animal was killed for examination.

Autopsy.—With the exception of one small hemorrhagic spot in the lungs nothing abnormal was found.

Notwithstanding this apparently negative finding, however, the blood, as well as emulsions of the liver and kidney of this animal, were inoculated into six normal guinea pigs, the blood into one and the mixed emulsions of the liver and kidney into five. The results were of doubtful nature with respect to positive transmission, although two of these animals showed some punctiform hemorrhages in the lungs when examined after 14 days.

Guinea Pig 8 (*Chart 8*).—This experiment was a duplicate of the experiment with Guinea Pig 7; that is, the animal was inoculated with the blood taken on

Dec. 19, 1919. As Chart 8 shows, the temperature rose to 104° F. in the afternoon of the 4th day, remaining high for the following 24 hours. It fell to 100.5° on the 6th day, when it seemed advisable to make a transfer.

Autopsy.—The examination showed a few hemorrhagic foci in the lungs, highly congested liver and kidneys, and a few hemorrhagic areas in the mucosa of the intestines.

Transfers with the blood and organ emulsions (liver and kidneys) of Guinea Pig 8 were made to several normal guinea pigs.

Transfers from Guinea Pig 8 (Second Generation).—

Guinea Pig 20 (Chart 9).—Dec. 24, 1919. Inoculated with the citrated blood from Guinea Pig 8, 1 cc. intraperitoneally. The temperature rose suddenly to 105° on the 5th day and remained at that point during the day. It fell to 103° the next day and to 101° on the 8th day. At this period the scleras seemed slightly icteric. The urine diminished to less than 2 cc. and contained albumin. The condition remained unchanged for the 3 succeeding days, the urine still containing albumin. Casts appeared on the 10th day. The yellowness in the conjunctiva was definite on the 11th day, and the animal died in the afternoon after subnormal temperature.

Autopsy.—Small hemorrhagic and congested areas in the lungs; liver smaller in size (?) and reddish brown with yellow mottles; kidneys unusually pale, and the demarcation between cortex and medulla indistinct. The spleen was somewhat enlarged, perhaps owing to secondary infection, and granular. In the liver and kidney stained by Levaditi's method fairly numerous leptospiras were demonstrated.

Guinea Pig 21.—Dec. 24, 1919. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions of Guinea Pig 8. Died of a secondary infection, but showed the presence of a mild leptospira infection, with albumin and casts in the urine.

Guinea Pig 22.—This experiment was a duplicate of the foregoing experiment. Temperature 103° F. on the 4th day, and fever continued for several days. Jan. 5, 1920. The animal was killed for examination.

Autopsy.—The lungs showed old hemorrhagic areas, but the other organs seemed normal.

The remainder of the citrated blood of December 19 was preserved in the ice box (about 10°C.) and after 5 days was inoculated intracardially into two normal guinea pigs, with negative results.

Second Specimen of Blood, Drawn on the 3rd Day of the Disease, December 20, 1919.

1 cc. of the citrated blood drawn on December 20 was inoculated intraperitoneally into three guinea pigs (Nos. 9, 10, and 11) on the same day.

Guinea Pigs 9 and 11.—These two animals had suggestive and almost identical temperature curves.

Autopsy.—When killed for examination on the 15th day, there were several foci of recent hemorrhage in the lungs, and the kidneys were congested; the other organs appeared normal.

Guinea Pig 10 (Chart 10).—This animal presented a more definite febrile reaction. On the 14th day it had a normal temperature and appeared well. It was killed for examination on that day.

Autopsy.—There were numerous hemorrhagic foci in the lungs; the abdominal wall and subcutaneous tissues were icteric and showed several large hemorrhagic areas. The adrenals were hemorrhagic, the kidneys highly congested, and the liver was apparently normal. There were pin-point hemorrhages in the gastric mucosa, and hemorrhages in the intestinal mucosa. The spleen appeared normal.

The remainder of the citrated blood of December 20 was preserved in the ice box (about 10°C.) for 4 days and 1 cc. was inoculated intracardially into each of two normal guinea pigs (Nos. 14 and 15).

Guinea Pig 14.—Sudden rise of temperature to 104° F. in the afternoon of the 4th day, but it dropped to normal (102°) on the next day. On the 6th day there was another rise to 103.5°, from which the temperature gradually fell to subnormal on the 8th day. The urine gradually diminished in quantity after the 4th day, and albumin and casts were present for several days. Ecchymotic hemorrhages were observed on the abdominal wall on the 8th day. There was a suspicion of jaundice. The animal rapidly recovered and was normal on the 13th day.

Guinea Pig 15.—Lost through secondary infection.

Third Specimen of Blood, Drawn on the 5th Day of the Disease, December 22, 1919.

The citrated blood from Case 2 drawn on December 22 was kept on ice for 48 hours (about 10°C.) before it was inoculated into two guinea pigs (Nos. 16 and 17) on December 24.

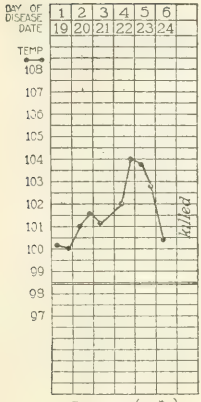


Chart 8 (GP#8).
Inoc. with 2nd day blood.

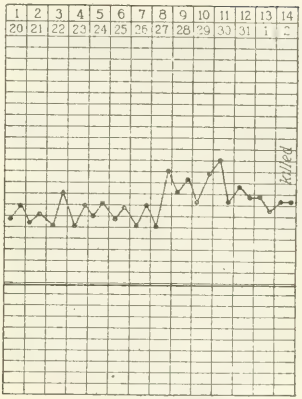


Chart 10 (GP#10).
Inoculated with 3rd day blood.

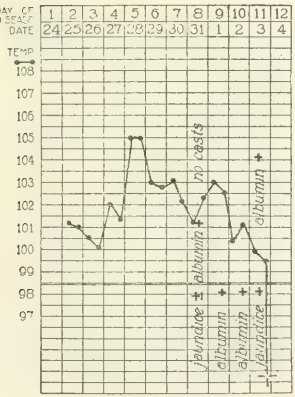


Chart 9 (GP#20)
Transfer from GP#8

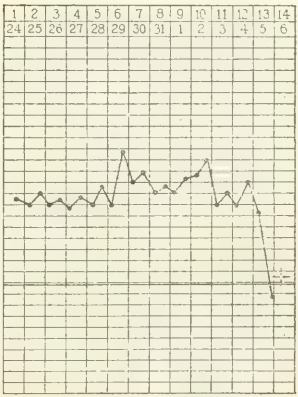


Chart 11 (GP#25)
Inoculated with 7th day blood

CHARTS 8 to 11. Direct transmission experiments with blood from Case 2.

Guinea Pig 16.—Showed a rise of temperature to 103° F. on the 4th day, a decline to 102.5° on the 5th day, and a rise again to 103.5° on the 6th. At this time the animal had congested conjunctivæ. On the 9th day the temperature became somewhat subnormal but soon returned to normal during the succeeding days. The animal was killed on the 13th day.

Autopsy.—A number of hemorrhagic foci were found in the lungs, the liver appeared very pale and brownish, the kidneys were somewhat congested, the spleen normal. The animal was probably recovering from an abortive infection.

Guinea Pig 17.—This animal had practically the same febrile reaction as Guinea Pig 16.

Autopsy.—Performed on the 13th day. The organs appeared normal, with the exception of a few hemorrhagic foci in the lungs.

Fourth Specimen of Blood, Drawn on the 7th Day of the Disease, December 24, 1919.

The citrated blood was intracardially inoculated into three guinea pigs (Nos. 24, 25, and 26) within 3 hours from the time of withdrawal, each animal receiving 0.2 to 0.5 cc.

Guinea Pigs 24 and 25 (*Chart 11*).—Both animals had a suggestive temperature curve but had no jaundice at any period.

Autopsy.—The lungs showed some suspicious recent hemorrhagic areas; the kidneys were rather congested. The other organs were normal. Results inconclusive.

Guinea Pig 26.—Perhaps died of secondary infection.

Transmission Experiments by Means of Cultures from the Blood of Case 2.

Cultures were made with the citrated blood of Case 2 by introducing into a series of six tubes containing the necessary culture media[11] quantities of the blood varying from 0.1 to 0.5 cc. and adding to each a layer of paraffin oil. The tubes were kept for several days at a temperature of 26–28°C. Then, since the leptospira grows only in the presence of oxygen, the upper layer of the semisolid medium was removed from each tube by means of a sterile pipette and the contents of two or more tubes were mixed in a Petri dish. Several guinea

[11] This consisted of a rabbit serum diluted three times with 0.9 per cent sodium chloride solution and rendered semisolid by the addition of 0.1 to 0.3 per cent nutrient agar (melted). Each tube contained about 6 cc. of the mixture.

pigs were inoculated with the same material. For every series of cultures made from any given specimen of blood at least two sets of guinea pigs were inoculated, and three sets if there was a larger number of tubes.

The cultures set up with the specimens of blood taken on five occasions (December 18, 19, 20, 22, and 24) remained for the most part free from any secondary contamination during the first 5 days. Shortly afterwards, however, darkening of the hemoglobin in several tubes indicated the presence of some sort of contamination, and microscopically a fungus growth was found. Unless a surface growth of fungus was already evident, however, the contents of these tubes were also used for inoculation, though tubes showing bacterial contamination were discarded. Such contamination was rarely met with in experiments with the blood of Case 2, and that occasionally occurring was probably due to technical error. Following are the results of animal inoculations with the various culture series.

Culture, First Series. Blood Drawn on the 1st Day of the Disease, December 18, 1919.

Dark-field examination of Tubes 1 and 2, 7 days after the cultures had been set up, revealed no organism. The contents of these two tubes (2 cc. of the uppermost layer of medium from each) were mixed together in a Petri dish, and 1 cc. of the mixture was inoculated intraperitoneally into each of three normal guinea pigs (Nos. 36, 37, and 38). No definite reaction followed the inoculations in Guinea Pigs 36 and 38, which, on examination 15 days after inoculation, presented nothing remarkable in any organ except for a congestion of the lungs.

Guinea Pig 37.—Slight rise in temperature on the 7th day (102.5° F. a.m., 103° p.m.), with return to normal the next day. On the 14th day the temperature rose suddenly to 105° a.m. and 103° p.m.; on the 15th day it was 102.6° a.m. The animal was killed the same day for examination.

Autopsy.—The lungs were typical, showing numerous hemorrhagic foci, but there was no other change.

Dark-field examination of Tubes 3 and 4 of the same series demonstrated the presence of a few fungi on December 25, 7 days after

inoculation. The uppermost layers of media from the two tubes were mixed and 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 39 and 40) on that day (December 25), with negative results.

Culture, Second Series. Blood Drawn on the 2nd Day of the Disease, December 19, 1919.

Five culture tubes were available for these experiments. These were divided into two groups, Tubes 1 and 2, and Tubes 3 to 5. Dark-field examination of these tubes was made 6 days after the inoculation. Tubes 1, 2, 3, and 5 showed no organism; Tube 4 showed a few immotile leptospiras, some apparently degenerating. Occasional coarse, short filaments (fungus) were also found. The number of leptospiras was so small that a repeated, thorough search with a powerful illumination was necessary to find one. The results of animal inoculation follow.

The contents (uppermost layers) of Tubes 1 and 2 of the second culture series were taken out and mixed. 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 41 and 42) on December 25 and into two more on December 26. Two died of secondary infection; the others remained well.

The uppermost layer was taken out of Tubes 3, 4, and 5 of the second culture series, and 1 cc. of the mixture of these, which had been found by dark-field examination to contain a few leptospiras, was inoculated intraperitoneally into each of three guinea pigs (Nos. 43, 44, and 45) 6 days after the cultures had been made. The results of these inoculations were all positive, as the protocols show.

Guinea Pig 43 (*Chart 12*).—The temperature rose to 103° in the morning of the 5th day. During the next 36 hours it fluctuated between 102° and 102.5°. On the 7th day it rose to 105.2° a.m. and 103.5° p.m. The amount of urine was still undiminished. On the 8th and 9th days the morning temperature was 102° and the evening 104°; on the 10th it was 102.5° in the morning and 103.4° in the evening; on the 11th it fell rapidly to normal. The animal was decidedly jaundiced. The urine diminished daily after the 9th day, and only 3 cc. were secreted during the 24 hours from Jan. 3 to Jan. 4, 1920. Albumin and casts were present. The animal was killed for examination and transfer on the 11th day.

Autopsy.—Findings typical. Intense jaundice; extensive subcutaneous ecchymoses; lungs distinctly hemorrhagic; gastrointestinal mucosa congested with

hemorrhagic areas; liver yellowish; kidneys hemorrhagic and yellowish; spleen somewhat enlarged.[12] Dark-field examination revealed the presence of leptospiras in the blood as well as in emulsions of the liver and kidney.

Transfers from Guinea Pig 43.—Blood from the heart and a mixture of emulsions of the liver and kidney were intraperitoneally inoculated into six normal guinea pigs, the blood into Nos. 106 and 107, and the emulsion into Nos. 108, 109, 110, and 111. The protocols follow.

Guinea Pig 106.—Jan. 4, 1920. Inoculated with 1 cc. of the citrated blood from Guinea Pig 43. Temperature 104° F. on the 3rd day, fell to normal on the 4th, rose to 104° a.m., 104.5° p.m. on the 5th, remaining high for another 24 hours. On the 7th day it was 103.5° a.m. and 102.2° p.m. Jaundice and albuminuria were both distinct. On the 8th day died after subnormal temperature.

Autopsy.—All the characteristic lesions, together with signs of a secondary infection with the paratyphoid bacillus (enlarged spleen and fibrinous exudate in the peritoneal cavity).

Guinea Pig 107 (*Chart 13*).—This experiment was a duplicate of the foregoing experiment. As shown in Chart 13 the course of the infection was typical, the temperature rising to 105.2° a.m. and 104.6° p.m. on the 5th day. On the 7th day the animal was intensely yellow, with albumin and casts in the urine. It was killed for examination on the 7th day.

Autopsy.—Findings typical. The blood and organ emulsions showed a few leptospiras.

Guinea Pig 108.—Jan. 4, 1920. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions from Guinea Pig 43. This animal suffered from a secondary paratyphoid infection and showed a temperature of 104.6° in the afternoon of the 3rd day. This early fever endured for 3 days longer. On the 7th day it fell to 103.5°. On the 8th day it reached normal, and the animal died after subnormal temperature on the 9th day. No jaundice developed.

Autopsy.—Several hemorrhagic foci in the lungs. No leptospira was found.

Guinea Pig 109.—This animal must have been suffering from an intercurrent infection before the inoculation, as the temperature was 103.4° in the afternoon of Jan. 3, 1920, a day before the inoculation. After inoculation the temperature fluctuated between 102° a.m. and 102.5° p.m. during the 2 days that followed. On the 4th day it was normal. On the 5th it rose to 103.5° a.m. and 103.2° p.m., on the 6th it was 102.5°, on the 7th 101.5° a.m. and 96° p.m. Jaundice developed on the 6th day.

Autopsy.—Performed on the 7th day. The lesions were typical, hemorrhages in the lungs, stomach, and intestines, kidneys highly hyperemic with minute hemorrhages, liver yellowish. The spleen was enlarged, showing the effect of a

[12] Blood culture showed the presence of the paratyphoid bacillus.

secondary infection with the paratyphoid bacillus. The blood and organ emulsions contained leptospiras. A blood culture from the heart showed the presence of the paratyphoid bacillus.

Guinea Pig 110 (*Chart 14*).—Duplicate experiment. This animal escaped secondary infection with the paratyphoid bacillus and ran a typical course of the leptospira infection. The temperature rose to 103° in the afternoon of the 3rd day, was 102.2° a.m. and 104° p.m. on the 4th, 103.8° a.m. and 104.6° p.m. on the 5th. From the 6th day the temperature rapidly fell and was 98° when death occurred in the morning of the 7th day. Jaundice was definite on the 6th day.

Autopsy.—Findings typical. The leptospira was demonstrated in the organ emulsions. The animal had epistaxis at the time of death.

Guinea Pig 111 (*Chart 15*).—Duplicate experiment. This animal also escaped secondary paratyphoid infection and ran a course of fever similar to that of Guinea Pig 110, except that the highest temperature never exceeded 104° (4th day). On the 5th the temperature was still 103.8°. The examination of the blood showed no leptospiras. The animal was killed for examination and culture in the afternoon of the 5th day. The citrated blood from the heart was examined carefully for the leptospira, and one was found after long search. This blood was used for cultivation, which in 7 days was successfully accomplished.

Autopsy.—Examination of the organs showed that jaundice had not yet developed. The lungs, stomach, and intestines were dotted with hemorrhagic foci. The liver was congested, with yellowish areas. The kidneys were congested. Emulsions of the liver and kidney contained a small number of leptospiras.

Guinea Pig 44 (*Chart 16*).—This is one of the three guinea pigs inoculated with 1 cc. of the mixture of Culture Tubes 3, 4, and 5 of the second culture series on Dec. 25, 1919. This animal, in striking contrast with the foregoing ones (Guinea Pig 43 and its transfers) showed a rather slowly developing infection without much febrile reaction. The temperature remained entirely within the normal fluctuation until the 7th day, and on the 8th day the morning temperature was slightly above normal (102.6° F.), rising to 103.6° in the afternoon. On the 9th day the temperature was normal in the morning and somewhat lower in the afternoon, and the animal was distinctly yellowish. On the 10th day the temperature registered 101.3° a.m. and 101.2° p.m., perhaps somewhat below the normal, and jaundice was intense. The animal died during the night.

Autopsy.—Performed in the morning, Jan. 4, 1920. Intense jaundice. There were extensive subcutaneous hemorrhagic foci on the abdominal wall and in adjacent regions. The lungs were yellowish, with numerous hemorrhagic areas, and the heart was dilated and contained dark clot and blood. The liver was yellowish brown, mottled with congested areas, the gall bladder empty, the kidneys were swollen, hemorrhagic, and degenerated, deeply bile-stained, and the adrenals hyperemic. The stomach contents were bloody, and there were ecchymoses in the mucosa. The intestines contained bloody stools; the mucosa was congested and showed many hemorrhagic spots. The spleen was normal. The

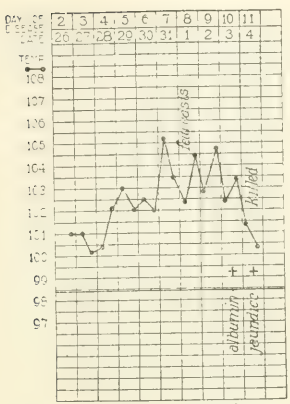


Chart 12 (GP#43).
Inoc. with culture of 2nd day blood

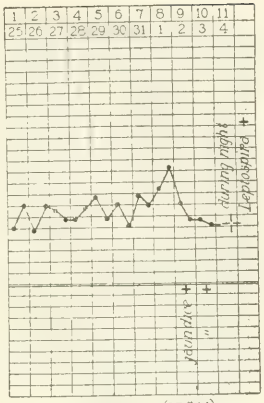


Chart 16 (GP#44).
Inoc. with culture of 2nd day blood

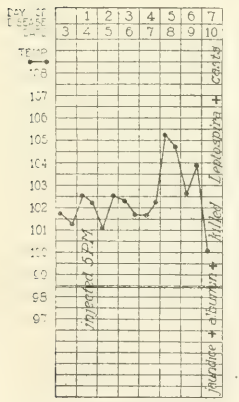


Chart 13 (GP#107).
Transfer from GP#43

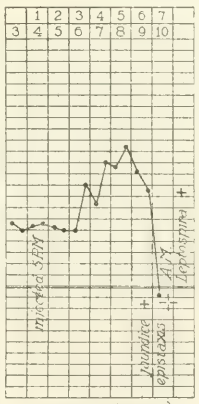


Chart 14 (GP#110)
Transfer from GP#43

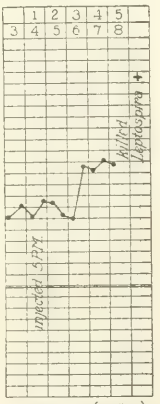


Chart 15 (GP#111)
Transfer from GP#43

CHARTS 12 to 16. Transmission experiments with cultures from Case 2.

bladder was almost empty (anuria); ovaries and uterus congested. In the scanty urine removed from the bladder there were albumin and numerous casts with erythrocytes. The leptospira was found in the blood.

Transfer with a mixture of the liver and kidney emulsions from Guinea Pig 44 was made into Guinea Pig 105 on January 4, 1920. The result was positive in every respect. The animal succumbed to a typical leptospira infection on the 7th day, and the leptospira was detected in the blood and organs.

Guinea Pig 45.—This is the last of the three guinea pigs (Nos. 43, 44, and 45) inoculated with the same material (culture made with the blood drawn on the 2nd day) on Dec. 25, 1919. The result of the inoculation was likewise positive, although the temperature curve deviated considerably from the curves of the other two animals, owing probably to a secondary infection with another organism. On the 11th day the animal had been running a temperature of 103–104° F. for 3 days. It was killed for examination on the 11th day.

Autopsy.—The lesions were typical except for the absence of distinct jaundice. The liver was, however, somewhat yellowish. The leptospira was not found by routine dark-field examination.

Culture, Third Series. Blood Drawn on the 3rd Day of the Disease, December 20, 1919.

The citrated blood of Case 2 drawn on December 20 was introduced into five culture tubes and incubated for 5 days at 26°C. Tubes 1, 2, and 5 were free from any contamination, while Nos. 3 and 4 showed a fungus growth. A search for the leptospira by dark-field examination showed occasional immotile leptospiras in the contents from Tube 4, but was unsuccessful in the case of the other tubes.

The uppermost layer of culture medium from Tubes 1 and 2 was taken out and the two were mixed, as was done also with Tubes 3, 4, and 5. The two mixtures were inoculated into corresponding groups of guinea pigs.

Guinea Pigs 46 and 47.—Inoculated with 1 cc. of the mixture of Culture Tubes 1 and 2. Both animals had a suggestive temperature curve. On the 13th day both guinea pigs were killed for examination.

Autopsy.—Findings similar, both animals having old hemorrhagic foci in the lungs, congested stomach and kidneys, and normal spleen. The liver was slightly yellowish in Guinea Pig 46.

There was perhaps an abortive infection in both these animals.

Three guinea pigs (Nos. 48, 49, and 50) were inoculated each with 1 cc. of the mixture of Culture Tubes 3, 4, and 5. The results were highly suggestive of an abortive leptospira infection with Guinea Pigs 48 and 49, and quite definite with Guinea Pig 50, in which jaundice was also present. From Guinea Pig 48 a positive transfer to another guinea pig was also accomplished.

Guinea Pig 48.—Typical temperature rise on the 5th day, reaching 103.8° F. a.m. and 103.4° p.m. The temperature remained normal on the 6th day and continued so until the 9th day, when the animal was killed for examination and transfer.

Autopsy.—The lesions found were small, discrete hemorrhagic foci in the lungs, swelling and congestion of the kidneys, and congestion of the stomach mucosa. The other organs appeared to be normal.

Transfers were made with the citrated blood from the heart of Guinea Pig 48 into two guinea pigs and with a mixture of the liver and kidney emulsions into four guinea pigs. One of the animals inoculated with the blood showed a temperature of 102.8°F. in the afternoon of the 8th day and 103° in the morning of the 9th day, but soon returned to normal. When it was killed for examination on the 12th day there were a few hemorrhagic patches in the lungs, the liver was somewhat yellowish, and the kidneys were congested.

Transfer with the organ emulsions from Guinea Pig 48 gave a more decided picture of the leptospira infection in some but no reaction in others.

Guinea Pig 98.—Temperature of 102.8° on the 5th day, 102.5° a.m. and 103.2° p.m. on the 6th, 103° a.m. and 102° p.m. on the 7th. On the 9th the temperature became normal. The animal was killed on the 12th day for examination.

Autopsy.—The lungs showed many recent hemorrhagic patches, the mucosa of the stomach several old hemorrhagic foci. The kidneys were pale; the spleen was normal.

Culture, Fourth Series. Blood Drawn on the 5th Day of the Disease, December 22, 1919.

Only four tubes were made with the citrated blood drawn on December 22 from Case 2, and after 9 days only two tubes appeared to be free from bacterial contamination. These tubes also contained a slight fungus growth which did not change the appearance of the

medium to any marked extent. Under the dark-field microscope two undoubted immotile leptospiras were found in the mixture of the contents of the two tubes. 1 cc. of the mixture was also inoculated into each of four guinea pigs.

The results of this series of inoculations were unsatisfactory, owing to a secondary infection in all the guinea pigs.

Culture, Fifth Series. Blood Drawn on the 7th Day of the Disease, December 24, 1919.

Six culture tubes were inoculated with the citrated blood of this date. Unfortunately the tubes were covered with an accidentally contaminated paraffin oil, and within a few days incubation at 28°C. all of them contained a profuse growth of fungus.

SUMMARY.

Injections into guinea pigs of the blood and the emulsions of liver and kidney obtained at autopsy from a fatal case of yellow fever in Merida induced in some of these animals, after a period of several days incubation, a rise of temperature which lasted 1, 2, or more days. When killed for examination at this febrile stage the animals invariably showed hemorrhagic areas of various size, sometimes few and sometimes numerous, in the lungs, and also, though less constantly, in the gastrointestinal mucosa, together with general hyperemia of the liver and kidneys. In a guinea pig (No. 6) inoculated with the liver emulsion of Case 1 there was a trace of jaundice on the 9th day. Injections of the blood or liver and kidney emulsions from such animals into normal guinea pigs reproduced the febrile reactions and the visceral lesions. The majority of the animals which were allowed to live and complete the course of the infection rapidly returned to normal (within several days). Examinations of these surviving guinea pigs after 2 weeks revealed the presence of rather old hemorrhagic foci in the lungs.

In the course of further attempts to transfer the passage strain, a secondary infection by a bacillus of the paratyphoid group caused many deaths among the guinea pigs and resulted finally in the loss of the strain from Case 1.

Most of the cultures made with the heart's blood taken at autopsy from Case 1 proved to be contaminated with a bacillus of the *coli* group. The contents of the apparently uncontaminated tubes were inoculated into guinea pigs, but the results were for the most part negative or vitiated by a secondary infection.

Dark-field search for the leptospira with the autopsy materials was negative, although prolonged and thorough examination was not practicable at the time of these experiments. Our efforts were concentrated on obtaining positive animal transmission rather than on the time-consuming demonstration of the leptospira, which when unsuccessful does not necessarily exclude the presence of the organism in small numbers. Likewise, the dark-field work with the material from guinea pigs was confined to a brief examination and was omitted in many instances. Under these circumstances no leptospira was encountered in any of the material from Case 1.

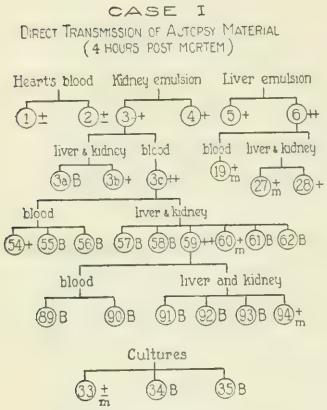
On the other hand, the results obtained with the specimens of blood from Case 2 were definitely positive, not only in the transmission of the disease directly, or indirectly by means of cultures, into guinea pigs, but also in the demonstration of the leptospira in the primary cultures and in the blood and organ emulsions of guinea pigs experimentally infected with such cultures.

Definite positive direct transmissions were obtained with the specimens of blood drawn on the 2nd and 3rd days. No blood was taken on the 4th or 6th days. There were indications of abortive or mild leptospira infection in the guinea pigs inoculated with the blood taken on the 5th day.

Regarding the inoculation of cultures from Case 2, it may be stated that only the cultures (leptospira +) made with the blood drawn on the 2nd day caused a definite fatal infection in guinea pigs. From this series a continuous passage in the guinea pig has been successfully accomplished. One of the guinea pigs (No. 48) inoculated with the culture 5 days old (leptospira +) made from the blood taken on the 3rd day presented typical symptoms, and a positive transfer from this to another animal (No. 98) was also made. Cultures of the blood drawn on the 5th and 7th days gave unsatisfactory results, owing to a secondary contamination. Leptospiras were detected in some of the culture tubes containing 2nd and 3rd day

specimens of blood from Case 2; they were few in number and for the most part immotile, owing perhaps to some unfavorable cultural condition such as a fungus contamination.

Charts 17, 18, and 19 give a summary of the experiments.



+ Temperature & lesions typical
++ All symptoms, including jaundice & lesions.
$^{+}_{m}$ Mixed infection.
± Doubtful leptospira infection
− Negative result in regard to leptospira infection.
$^{++}_{m}$ Severe leptospira infection with all symptoms, but concomitant secondary infection.
B Bacterial infection
L Leptospira.

Chart 17.

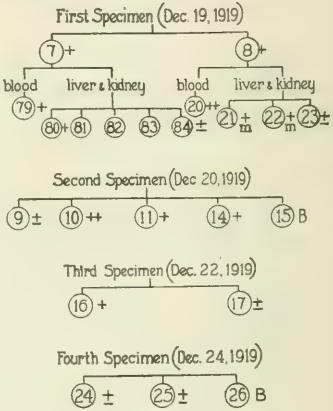


Chart 18.

CASE II

TRANSMISSION BY MEANS OF CULTURES FROM BLOOD

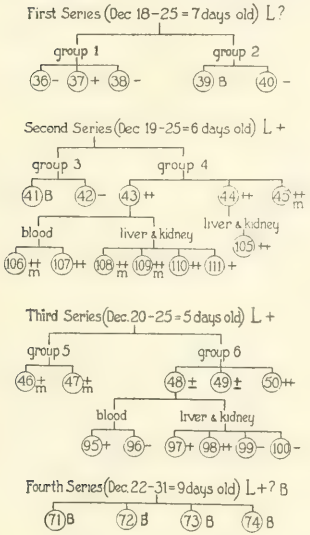


Chart 19.

[Reprinted from The Journal of Experimental Medicine, November, 1920, Vol. xxxii, No. 5, pp. 627–637.]

IMMUNOLOGICAL STUDIES WITH A STRAIN OF LEPTOSPIRA ISOLATED FROM A CASE OF YELLOW FEVER IN MERIDA, YUCATAN.

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Morphology, Cultural Properties, and Virulence of the Strain of Leptospira Isolated in Merida.

The morphological features and cultural properties of the strain of *Leptospira icteroides* isolated in Merida are similar to those of the Guayaquil strains.[1] With respect to pathogenicity and virulence we obtained the following data.

Guinea Pig 112 was inoculated on January 4, 1920, with the mixed emulsions of liver and kidney from Guinea Pig 43, which had typical symptoms as a result of inoculation of a culture made with blood drawn from Case 2 on December 19, 1919. No. 112 came down with typical symptoms on the 8th day and was killed for transfer on January 12. The liver and kidney were emulsified together with 0.9 per cent saline solution, about 1 gm. of organ material to 10 cc. of saline solution. The emulsion was allowed to stand several minutes until the supernatant fluid was free from coarse tissue particles. The clear portion, which under the dark-field microscope showed a few leptospiras in every field, was used to determine the minimum lethal dose for guinea pigs. The procedure consisted in inoculating intraperitoneally graduated amounts of the emulsion into as many guinea pigs as the number of dilutions required, each amount being contained in a uniform volume of 1 cc. of 0.9 per cent saline solution. The results of the experiment are recorded in Table I.

The period of incubation varied from 3 to $7\frac{1}{2}$ days, and death occurred 7 to 10 days after the time of inoculation. The duration

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

of illness, calculated from the beginning of fever until death, varied from $2\frac{1}{2}$ to $4\frac{1}{2}$ days. We did not succeed in finding the minimum lethal dose, which apparently lies below 0.0001 cc., the smallest quantity of emulsion employed in the present experiment.

The Merida strain showed a marked pathogenicity for young pups, 6 weeks old, when given intraperitoneally.[2]

TABLE I.

Determination of the Minimum Lethal Dose of the Merida Strain.

Guinea pig No.	Amount of organ emulsion.	Length of incubation.	Time of death.
	cc.	*days*	*days*
163	0.5	3	7
164	0.2	$3\frac{1}{2}$	8
165	0.1	$3\frac{1}{2}$	7
166	0.01	5	8
167	0.001	$7\frac{1}{2}$	10
168	0.0001	5	9

Dog 1.—Jan. 14, 1920. Inoculated with 4 cc. of the kidney emulsion from Guinea Pig 102 (second passage in guinea pigs) intraperitoneally. The temperature was 39.6°C. on the 4th day, falling rapidly to 38° the next morning. In the afternoon of the 7th day the animal was found near collapse (37°) and was killed for examination. Jaundice had been noticed in the conjunctivæ on the 6th day, and there was general jaundice on the 7th day. Albuminuria began on the 5th day and increased until the animal was killed.

Autopsy.—General icterus; marked congestion of the lungs and other organs. Hemorrhagic foci were found in small number in the lungs and intestinal mucosa. The kidneys were congested and degenerated. The liver was mottled with yellowish patches. No subcutaneous or intramuscular hemorrhages were found. The serous membranes were free from hemorrhage.

Dog 2.—This animal received the same material as Dog 1 at the same time. The temperature curve was somewhat more irregular, 39°C. on the 2nd and 3rd days, normal on the 4th day, and 39.6° on the 5th day. The decline was very rapid, 37.5° a.m. and 36.2° p.m. on the 6th day. The animal died during the night. Albuminuria was present on the 4th day. Jaundice was noticeable on the 5th day and had definitely increased during the 6th day.

Autopsy.—General jaundice; liver and other organs yellowish. Few small hemorrhagic foci in the lungs. The stomach contained some digested blood,

[2] Villamil Mendoza, M., Apuntes acerca de la fiebre amarilla, Thesis, Merida, 1920.

and the mucosa of the intestines was profusely hemorrhagic. The kidneys were congested and degenerated. Subcutaneous tissues, muscles, and serosa not hemorrhagic.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

The identity of the Merida strain of the leptospira was established by means of a series of experiments in which varying quantities of anti-*icteroides* serum were injected, together with 1 cc. of an emulsion of the liver and kidney from Guinea Pig 43, into several normal

TABLE II.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

Guinea pig No.	Amount of serum.	Result.	Lesions.
	cc.		
109	0 (control).	Killed after 6 days.	Typical.
110	0 (").	Died " 6 "	"
111	0 (").	Killed " 4 "	" lung lesions.
112	0.1	Survived.	
113	0.01	"	
114	0.001	"	
115	0.0001	"	

guinea pigs. The anti-*icteroides* serum had been prepared in a horse by repeated injections of different strains of *Leptospira icteroides* from Guayaquil cases. The results are recorded in Table II.

The outcome of this experiment made it evident that the Merida strain belongs to the same group as the Guayaquil strain.

Polyvalent anti-*icteroides* immune serum prepared in the horse or monovalent anti-*icteroides* immune serum prepared in the rabbit had a definite devitalizing action upon the Merida strain, while antiserums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serotherapeutic Experiments.

The purpose of the next series of experiments was to ascertain whether or not the same immune serum possessed a therapeutic value in infection with the Merida strain such as had been demonstrated in the case of experimental infection in guinea pigs with the Guayaquil strains.[3] Thirty-one guinea pigs were inoculated at the same time with 0.5 cc. of the organ emulsion, representing at least 5,000 minimum lethal doses. 1 hour after inoculation two of the animals were given an injection of 0.0001 cc. of the serum, and two more received an injection of 0.1 cc. The procedure was continued with each group of four animals after periods of 24, 48, and 72 hours, and 4 and 5 days, respectively. After 5 days the amounts of serum injected were increased to 1 and 2 cc., as it had been found that the smaller doses had no effect at this period of the disease.

All the animals which received no serum until after 72 hours showed fever at that time; in those untreated until 4 days there were fever and slight jaundice; those not treated until 5 days showed a decline in temperature and increasing jaundice; and in the group which received the injection after 6 days there were intense jaundice and subnormal temperature, and the animals were near or in collapse. The results of this series are summarized in Table III.

0.1 cc., therefore, of the anti-*icteroides* serum prevented any external manifestation of the infection if given before the onset of fever (within 72 hours after inoculation). The same dose, given to animals in the febrile stage, but without jaundice, prevented the development of jaundice; animals still having fever and showing more or less jaundice at the time of injection of this dose likewise survived, and in some jaundice rapidly disappeared in 24 hours. On the other hand, this quantity of serum failed to prevent a fatal outcome when given to animals in which defebrescence and increasing jaundice had set in. The nephritic symptoms, which had existed since 72 hours after inoculation, were rapidly increasing by this time.

0.0001 cc. of the serum prevented a fatal infection in three out of four guinea pigs when given within 24 hours. In one animal which

[3] Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 159.

died from the typical infection there was a prolongation of the incubation period (6 days) and the duration of illness ($5\frac{1}{2}$ days). In two of the three surviving animals fever developed after 4 days, but they recovered without showing jaundice at any time. Injection of this quantity of serum 48 hours after inoculation, or later, had no effect upon the course of the disease, all animals so treated dying within 5 to $8\frac{1}{2}$ days with typical symptoms.

Seven guinea pigs which were near or in a state of collapse 6 days after the inoculation were given 1 cc. (three animals) and 2 cc. (four animals), but with one exception all died within $\frac{1}{2}$ to $2\frac{1}{2}$ days from the time of injection of the serum.

It may therefore be concluded that the anti-*icteroides* serum here employed is able, in a dose of 0.1 cc., to protect a guinea pig from a fatal infection against at least 5,000 minimum lethal doses of the Merida strain when administered during the incubation period or at any early stage of the disease (fever and beginning of jaundice), but that it has almost no effect upon the course of the disease if given at a later period when the animals are deeply yellow and the temperature has begun to go down. At or near collapse even 1 or 2 cc. failed to prevent death in the majority of animals. On the other hand, a quantity as minute as 0.0001 cc., when given within 24 hours, is able to protect the majority of animals from a fatal infection.

If man's degree of susceptibility to *Leptospira icteroides* is comparable to that of the guinea pig, it may reasonably be assumed that the injection of the anti-*icteroides* serum at an early period of the disease will have a beneficial effect similar to that observed in the treatment of the experimental infection in guinea pigs. On the basis of body weight a man of 80 kilos would require about 200 times the amount of serum needed to save a guinea pig of 400 gm.; that is, 20 cc. (0.1 cc. $\times$ 200 = 20 cc.). The mode of administration should be intravenous, and if necessary the injection should be repeated at short intervals (4 hours).

TABLE III.

Serotherapeutic Value of the Anti-icteroides Serum against 0.5 Cc. (5,000 Minimum Lethal Doses) of an Organ Emulsion, Merida Strain.

Length of time before serum injection.	Symptoms.	Amount of serum injected.			
		0.0001 cc.	0.1 cc.	1 cc.	2 cc.
hrs.					
1		No. 131. Incubation period 6 days. Died after 11½ days.	No. 133. No infection.		
		No. 132. No infection.	" 134. " "		
24		" 135. Fever from 6th day on, but recovered.	" 137. " "		
		No. 136. Fever on 5th day, but recovered.	" 138. " "		
48		No. 139. Died after 6½ days.	" 141. " "		
		No. 140. Died after 5 days.	" 142. " "		
72	Fever; no jaundice.	No. 143. Died after 7½ days.	" 145. Survived without jaundice.		
		No. 144. Fever on 5th day. Died after 7½ days.	No. 146. Survived without jaundice.		

days					
4	Fever; jaundice.	No. 147. Died after 8½ days. No. 148. Died after 6 days.	No. 149. Survived, but jaundice increased next day. No. 150. Survived; jaundice disappeared next day.		
5	Temperature down; jaundice.	No. 151. Died after 6 days. No. 152. Died after 5½ days.	No. 153. Died after 8½ days. No. 154. Died after 7½ days.		
6	Near or in collapse.			No. 159. Died after 7½ days. No. 160. Died after 8½ days. No. 161. Died after 8 days.	No. 155. Died after 8½ days. No. 156. Died after 6½ days. No. 157. Died after 6½ days. No. 158. Recovered.

Pfeiffer Phenomenon with the Serums of Yellow Fever Convalescents in Merida.

Pfeiffer's phenomenon with *Leptospira icteroides* had been previously observed with a limited number of serums from yellow fever convalescents in Guayaquil[4] and was positive in about 85 per cent of the cases studied. It was desirable that this line of observation should be extended to yellow fever cases existing elsewhere, and we availed ourselves of the present opportunity with the Merida cases. Our first intention was to study as many specimens of serum from yellow fever convalescents in Merida as could be obtained, with as many strains of cultures of *icteroides* as possible. This plan could not be carried out, however, for two reasons; there were only a few convalescents accessible, and all the Guayaquil cultures brought down by us to Merida were lost within a few days after our arrival, and before our work had been started, by an accidental rise of temperature for 3 days to 56°C. of the thermostat in which the cultures had been placed for safety. Hence work on the Pfeiffer phenomenon was possible only after the isolation of *Leptospira icteroides* from Case 2.

There were two convalescents at the yellow fever hospital (Casa de Salud), Cases 2 and 3.[5] Both patients (soldiers) were infected in the same house in Merida, Case 3 preceding Case 2 by perhaps 2 weeks. The serum for the Pfeiffer test was drawn at the end of the 5th week of the disease in Case 3 and during the 3rd week in Case 2. Both specimens were still distinctly jaundiced. Through the courtesy of Dr. A. Lara we were given specimens of serum from two other convalescents who had had a typical attack of yellow fever in August, 1919; that is, about 5 to 6 months previously. One was a young woman, whose urine still contained albumin at that time, but who showed no bile pigment either in the urine or in the serum.

The following procedure was employed. 0.5 cc. of each specimen of serum was mixed with 1.5 cc. of a pure culture of the Merida strain of the leptospira and the whole injected into the peritoneal cavity of a guinea pig. The exudate was withdrawn from the peritoneal cavity after 30 minutes for dark-field examination. Table IV is a record of the results.

[4] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.
[5] Noguchi, H., *J. Exp. Med.*, 1920, xxxii, 601.

The serums from yellow fever convalescents in Merida gave uniformly a positive Pfeiffer reaction when tested with a culture of the leptospira isolated from Case 2. Although the guinea pig inoculated with the mixture of this culture and the serum from the same patient died of secondary infection too soon to permit any observation with regard to the power of the serum to protect the animal from a fatal infection, the animal which received the serum from Case 3 with this culture escaped the infection altogether. It may be supposed that this patient had been attacked by the same strain that later infected the other two soldiers (Case 1, fatal, and Case 2) living in the same

TABLE IV.

Pfeiffer Reaction with Serum from Convalescents and the Merida Strain.

Serum No.	Type of infection.	Length of time after onset of disease.	Pfeiffer reaction.	Remarks.
		wks.		
1 (Case 2).	Moderately severe.	3	Positive.	Died of intercurrent infection within 24 hrs.
2 (" 3).	Moderately severe.	5	"	No infection resulted.
		mos.		
3 (civilian).	Typical.	5	"	Died after 7 days with typical infection.
4 (").	"	6	"	Died after 7 days with typical infection.
No serum (control).			Negative.	Died after 9 days with typical infection.

house, hence such complete protection. On the other hand, Serums 3 and 4 were from two civilians who had recovered from yellow fever at least 5 months previous to the time when their blood was tested. These serums gave a positive Pfeiffer phenomenon but failed to protect the animals.

These four serums were brought back to The Rockefeller Institute to be tested with the Guayaquil strains of *Leptospira icteroides* and also with some of the strains of *Leptospira icterohæmorrhagiæ*. The serums from Cases 2 and 3 were accidentally lost during transportation. Moreover, during the journey the remaining two had to be

kept at a comparatively high temperature (heated steamer cabin and trains) for 15 days, and their activity must have suffered considerably. Nevertheless, tests were made on March 5; that is, 2 months from the time of collection. The amount available of Serum 3 was 0.75 cc. and that of Serum 4, 0.9 cc. Each specimen was divided into three equal parts and tested with two Guayaquil *icteroides* strains (Nos. 1 and 5) and with the American No. 1 strain of *icterohæmorrhagiæ*. The results obtained are presented in Table V.

TABLE V.

Pfeiffer Reaction with Serum from Convalescents Tested with Two Guayaquil Strains of Leptospira icteroides and One Strain of Leptospira icterohæmorrhagiæ.

Serum No.	*Leptospira icteroides.*		*Leptospira icterohæmorrhagiæ.*
	Guayaquil Strain 1.	Guayaquil Strain 5.	American Strain 1.
3	Positive.	Doubtful.	Negative.
4	Partial reaction.	Partial reaction.	"
No serum (control).	Negative.	Negative.	"

While no definite conclusions can be drawn from the results obtained with these old serums, it is possible to recognize an unmistakable specific reaction with the *icteroides* strains, particularly between Serum 3 and No. 1 of the Guayaquil *icteroides* strains. With Serum 4 there was not complete destruction of the *icteroides* organisms; but about one-half of them became paralyzed, and some were degenerated, although many active organisms were simultaneously present. There was no effect whatever upon American Strain 1 of *Leptospira icterohæmorrhagiæ*.

SUMMARY.

Identification of the leptospira isolated from a case of yellow fever in Merida was accomplished by means of an anti-*icteroides* immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides*. The immune serum showed a protective action of high titer against the Merida strain, thus establishing its efficacy as a therapeutic agent against this strain. Polyvalent anti-*icteroides*

immune serum prepared in the horse or monovalent anti-*icteroides* immune serums prepared in the rabbit had a definite devitalizing action upon the Merida strain, while immune serums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serums from yellow fever convalescents in Merida gave a positive Pfeiffer reaction with the Merida strain of *Leptospira icteroides*. The reactions between the Guayaquil strains (Nos. 1 and 5) and two of these serums from convalescents varied from definitely positive to doubtful, owing probably to the diminution of active immune principles in the serums during the prolonged and unfavorable conditions of their transportation.

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SYPHILITIC INFECTION OF THE CENTRAL NERVOUS SYSTEM OF THE RABBIT.

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Since it was first shown that rabbits were susceptible to infection with *Spirochaeta pallida*, numerous attempts have been made to devise means of producing an infection of the central nervous system in these animals which might be utilized in studying this phase of human syphilis. For the most part, the experiments which have been carried out appear to have been based on the assumption that this could not be accomplished by a simple testicular or scrotal inoculation, but that it was necessary to resort to some intensive form of inoculation, such as an intravenous, intracardial or intracranial inoculation or to the use of repeated inoculations of various kinds. This idea may be accounted for in part by the use of recently isolated strains of *Spirochaeta pallida* and in part by the apparent inability to produce a generalized disease in the rabbits by ordinary methods of inoculation.

How successful these efforts have proved it is difficult to say. The evidence of infection which has been submitted in cases other than those of direct inoculation into the cerebrospinal canal has consisted largely in the demonstration of lesions which, although analogous to certain lesions observed in man, might be produced by a variety of causes and are frequently found in rabbits which have not been inoculated with *Spirochaeta pallida*. The significance of the lesions described, therefore, has remained somewhat uncertain.

It appeared to us that, since a widespread dissemination of spirochetes always occurred in rabbits inoculated in the testicles or scrotum, whether lesions developed elsewhere or not, the first thing to be determined as an approach to the study of infections of the central nervous system was whether or not the organisms gained access to the cerebrospinal canal in ordinary types of infection and second, whether the same influences which control processes of infection in

other parts of the body might not extend also to localization of the organisms in the central nervous system.

It was obvious also that the first essential in attempting to determine these facts was the use of a strain of *Spirochaeta pallida* which was as well adapted to the rabbit as possible.

Accordingly, a small series of experiments was undertaken, the purpose of which was to determine the presence or absence of *Spirochaeta pallida* in the central nervous system at various periods of the experimental infection, more as a means of orientation than as an attempt to solve any particular problem. However, the results of these experiments throw considerable light on infection of the central nervous system in the rabbit, and for that reason may be reported in brief.

EXPERIMENTAL PRODUCTION OF SYPHILIS.

The method of procedure was to obtain cerebrospinal fluid from infected animals as free from contamination with blood or tissue juices as possible and to determine the presence of spirochetes by injecting this fluid into the testicles of normal rabbits. To accomplish this, the animal was killed with ether and bled from the heart to relieve the congestion of the meningeal and other vessels. The tissues over the occipito-atlantoid membrane were then removed and the membrane scraped clean, after which a needle was inserted through the membrane and the fluid removed in fractional amounts by suction with a small syringe. In this way, an average of 0.6 to 0.8 cc. of fluid could be obtained which was free from any visible contamination with blood.

The fluid thus obtained was examined microscopically and injected into one testicle of one or two normal rabbits, depending on the amount of fluid available. These animals were then kept under observation for a period of sixty days for the purpose of determining an infection in the testicle.

The spinal fluid of thirteen rabbits that had been inoculated in the testicles was examined by this method. Nine of these rabbits were infected with Nichols' strain of *Spirochaeta pallida* which was isolated from the cerebrospinal fluid of a neurorecidive in 1912 and four of them with an organism isolated from a mucous patch by Zinsser and Hopkins in 1913.

Results.

The Demonstration of Spirochetes.—The first series of animals examined consisted of five rabbits with early testicular infections. The fluid from one of these produced an orchitis after an incubation period of forty-three days in one of the two animals inoculated. Subinoculations from the others were all negative at the end of sixty days.

The successful transfer in this case came from an animal with an orchitis of eight days' duration which was produced by the Nichols strain of *Spirochaeta pallida*. There were large and actively progressing lesions in both testicles but no lesions elsewhere.

A second series of inoculations was then made from three rabbits with actively progressing generalized lesions. These gave two positive results. In one case, infection was again obtained in only one of the two subinoculations, while in the other, only one test animal was used.

One of the animals in which infection of the nervous system was proved had marked periosteal lesions with extensive necrosis of the nasal bones, which suggests the possibility of invasion of the central nervous system by way of the lymphatics. This animal was inoculated in both testicles with the Zinsser-Hopkins strain of *Spirochaeta pallida*, and the development of generalized lesions was induced by castration.

It is important to note that the incubation period of the infection produced by the spinal fluid of this animal was only thirty-six days, which is reasonably close to that of an infection produced by an inoculation with 0.5 cc. of heavily infected blood or with an emulsion from an infected lymph node.

The other animal was inoculated in one testicle with the Nichols strain of *Spirochaeta pallida* and subsequently given a dose of 5 mg. per kilo of arsenophenylglycyl dichloro-m-aminophenol as a means of suppressing the testicular reaction. Generalized lesions were just beginning to appear and there were slight lesions of the nasal splints when the animal developed signs of a meningitis. Although the spinal fluid contained an increased number of cells, it was clear and no spirochetes could be demonstrated by dark field examination. At necropsy, no abnormality of the brain or cord could be detected beyond a possible injection of the meningeal vessels and a slight clouding of certain areas of the meninges.

The exact incubation period of the infection produced with this fluid cannot be given. The lesion in the testicle was first discovered forty-two days after inoculation, but it was then a well developed lesion of at least three or four days' duration.

The third group of animals whose spinal fluid was examined was composed of five rabbits, all of which had developed generalized lesions. At the time the examinations were made, these lesions were either regressing or had completely disappeared so that this group of cases might be classed under the head of inactive or latent infections. Inoculations from all of these animals gave negative results.

Alterations in the Spinal Fluid.—In addition to the demonstration of spirochetes in the cerebrospinal fluid of three animals, mention should also be made of the occurrence of certain obvious abnormalities in the fluid of animals when no active infection could be proved.

The striking features of these changes consisted in a slight clouding of the fluid and the presence of flocculi which on microscopic examination proved to be due to an increased number of cells and the formation of aggregates of cells and granular detritus.

These conditions were practically constant in the animals of group three, which suggests an antecedent infection in these cases with a subsequent disappearance of the spirochetes such as occurs during remissions in other forms of localized infection. This possibility is supported by the apparent frequency with which spirochetes were demonstrated during an active period of infections of an analogous character.

CONCLUSIONS.

This series of experiments, although too limited to warrant any broad generalizations in regard to syphilitic infection of the central nervous system, is sufficient to show that such infections may be produced in the rabbit by an ordinary testicular inoculation of well adapted strains of *Spirochaeta pallida*, and that spirochetes may invade the central nervous system at a very early period of the infection.

How often this occurs is impossible to say since the methods used are subject to obvious limitations both as regards the type of infection which may be demonstrated in this way and the activity of the infection at the time the examination is made.

From this small series of experiments, it would appear, however, that invasion of the central nervous system by *Spirochaeta pallida* and the development of localized lesions are subject to the operation of the same set of conditions as are concerned in the occurrence of other manifestations of a generalized infection, and that by taking advantage of these conditions, it should be possible to favor this form of infection so as to increase both the incidence and severity of central nervous system involvement.

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SIMPLIFIED PRODUCTION OF ANTIMENINGOCOCCIC SERUM.

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Although a large amount of work in several countries has been done relative to the production of an effective antimeningococcic serum, the problems involved have not yet been wholly resolved. The increased demands arising from war conditions acted as a potent stimulus first to simplify manufacture and then to insure an effective product. This paper records primarily attempts to simplify the manufacture of an efficacious serum and deals incidentally with a number of still mooted questions regarding the antigenic properties of the meningococcus on which the production of such a serum largely rests.

It is now generally recognized that the meningococcus is not a simple, fixed antigenic entity but rather that the term meningococcus covers a class of closely related microorganisms, the distinguishing common characters of which relate to certain cultural and fermentative qualities and the power to set up in man particular forms of inflammation of the leptomeninges, while they differ markedly in their immunologic responses. Thus for identification the cultural properties are of first importance and for serum production the antigenic structure is paramount.

Ever since Dopter[1] first distinguished the immunologically distinct parameningococcus the classification of the meningococci has been under discussion and agreement has not yet been reached. Everyone admits the existence of two main groups called normal or regular meningococcus and parameningococcus; the disturbing factor is the

[1] Dopter, C., *Compt. rend. Soc. biol.*, 1909, lxvii, 74. Dopter and Pauron, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

occurrence of intermediate cocci which resemble either the regular meningococcus or the parameningococcus but are immunologically less sharply defined than are the pure types of the main groups. This fact has led to the setting up of main and subsidiary types as, for example, in Gordon's classification which recognizes four types of meningococci.[2] But Gordon's classification has not received general acceptation and for the reason that observers cannot agree on the two subsidiary type strains.

These considerations have far more than theoretical interest, since experience has shown that a therapeutically effective antimeningococcic serum should possess wide capacities of immunologic activity as measured by the agglutinin content. Since the early work on the subject by Flexner and Jobling[3] and the later studies of Amoss and Wollstein,[4] it has been the custom to employ for the immunization of horses a large number of cultures including representatives of the regular, the para, and many intermediate strains of the meningococcus. The number of strains used in the antigen might reach 50 or more, depending upon the reaction of the serum with meningococci derived from the cerebrospinal fluid of many cases of epidemic meningitis. When the test with such a culture showed low agglutination titer the strain was added to those used for immunization. The purpose of this was to produce a serum with as wide an agglutination index as practicable.

It is obvious that this procedure implies an empirical method. In endeavoring to simplify the manufacture of antimeningococcic serum it seemed worth while to determine more precisely than had yet been done whether a wide agglutination index could be obtained from a small number of antigenically different strains. Several facts had already rendered it probable that something in this direction was achievable. For example, it had been noted that the immunity response in the horse was wider than in the rabbit and also that the longer the injections of certain fixed cultures were continued, the more inclusive the agglutinin content became. In addition to these essential data there

[2] Gordon, M. H., *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

[3] Flexner, S., and Jobling, J. W., *J. Exp. Med.*, 1908, x, 141.

[4] Amoss, H. L., and Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 403.

was a still further important fact; namely, that approximately 80 per cent of all cases of epidemic meningitis arose from infection with the two type strains; that is, the regular meningococcus and the parameningococcus. Hence the production of an effective serum for this large proportion of cases of meningitis appeared to be relatively simple; the real problem was to make the serum effective against the 20 per cent of cases due to the highly variable subsidiary strains.

Fortunately, we possessed as a standard of comparison for the sera to be produced with a limited number of strains, samples of polyvalent sera, of established therapeutic efficacy, which had been produced at The Rockefeller Institute by the injection of 51 spinal cultures of the meningococcus selected in accordance with the method outlined above.[5] These 51 cultures were classified as follows: 10 were regular, 26 were para, and 15 were intermediate (subsidiary) strains of meningococcus.[6]

Horse Sera Produced with a Small Number of Strains.

When the experiments with horses with a smaller number of cultures of the meningococcus were begun in 1917 we had already observed that rabbits which were immunized with a single type strain over long periods of time yielded sera which contained agglutinins not only for that strain but also, in less amount, for heterologous strains including even those of the opposite type.

Use of Five Strains as Antigen.—The experiments described below were made on two horses (Nos. M 24 and M 25). Injections were begun with four cultures which were classed immunologically as

[5] The polyvalent serum issued for therapeutic purposes was composed of serum from at least three horses. Because of the variation in the response of different horses, it has been our practice to pool serum from several horses to insure a properly balanced product.

[6] In 1914 two strains of parameningococcus were brought from Dopter by a member of the staff of The Rockefeller Institute. It was on the basis of these strains that our classification was made. Recent comparison of our stock strains with sera and cultures lately obtained from England and France shows that our original classification is the reverse of the present accepted grouping. Conforming to general usage, we have revised our classification in this paper, so that the regular or normal group mentioned in previous papers from these laboratories is here designated as para and *vice versa*.

follows: Culture 1, para type strain; Culture 60, regular type strain; and Cultures 30 and 31 of different intermediate types. Serum obtained in early bleedings of these horses did not agglutinate Culture 38 (also an intermediate) which was then added to the antigen.

Experiment 1.—The method of immunization was as follows: After a glanders test and the subcutaneous injection of 1,500 units of antitetanic serum, Horses M 24 and M 25 were tested for sensitiveness to the meningococcus by the intravenous injection of minute doses (0.05 cc.) of a saline suspension (2.5 cc. to an agar slant culture). The immunizing injections were begun on November 29, 1917, living cultures being given in a total dose of 0.12 cc., and were continued according to the method described by Amoss and Wollstein.[4] Trial bleedings were made with Horse M 24 after 3, 10, and 14 months, and with Horse M 25 after 2, 10, and 14 months.

TABLE I.

Number of Stock Strains Agglutinated by the Serum of Horses M 24 and M 25 Immunized with Five Strains of Meningococcus.

Horse No.	Duration of immunization.	Agglutination test positive.	
		1:400 or higher.	1:100
	mos.		
M 24	3	35 of 51	50 of 51
" 24	10	43 " 60	59 " 60
" 24	14	51 " 56	56 " 56
" 25	2	35 " 58	40 " 58
" 25	10	32 " 60	58 " 60
" 25	14	47 " 56	56 " 56

In Table I the progress of the immunization is summarized according to the number of stock strains (cultures) which were agglutinated in a serum dilution of 1:100 and of 1:400 or higher. 51 of these stock strains were those used in the manufacture of the therapeutic polyvalent serum.

Table I indicates that by employing for purposes of immunization as few as five cultures, representing different strains, of the meningococcus, a serum is produced which shows considerable agglutinative capacity for as many as 51 selected stock cultures, including the

English type strains.[7] The titer of the serum for this large number of cultures equalled that previously required for a polyvalent serum prepared with a far larger number of cultures.

Use of Three Strains as Antigen.—In this experiment the strains were reduced to three, representing regular and para types.

Experiment 2.—The procedure with Horse M 31 was similar to that already described. The injections were begun on May 1, 1918, and trial bleedings were made after 5 and 9 months of immunization. Two regular cultures (Strains 60 and 79) and one para culture (Strain 85) were employed for the injection.

Table II summarizes the results obtained, which are the equivalent, for the same period of injection, of those obtained with the five strains as shown by Table I. Although thirteen of the stock cultures did not agglutinate in the 1:400 dilution after 9 months of immunization all were agglutinated in dilutions ranging from 1:50 to 1:200.

TABLE II.

Number of Stock Strains Agglutinated by the Serum of Horse M 31 Immunized with Three Strains of Meningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
5	32 of 50	47 of 50
9	43 " 56	55 " 56

Monovalent Horse Sera.

The total amount of meningococcic antigen which can be given a horse varies and is obviously limited. The use, therefore, of a single antigen might result in a greater antibody content for the homologous strain than could be obtained against the individual strains of a multiple antigen. Indeed, certain therapeutic monovalent sera have been prepared in this way by the Pasteur Institute and more recently by Gordon and others in England working under the Medical

[7] The English type strains were kindly supplied by Dr. Gordon.

Research Council.[8] The intent in both these instances was to fortify the therapeutic activity of the serum for treating cases of epidemic meningitis with type antisera after the type of infecting meningococcus had been determined.

It is not our intention in this place to discuss the practicability of this method of procedure or even to analyze the results thus far secured by the English investigators mentioned above. We would remark, however, that we are dubious as to the advisability of making a substitution of a monoserum for an active polyserum in view of the uncertainties and lack of uniformity which still surround the practical work of determining and distinguishing the so called types of meningococci. But as the data which follow show, the antigenic constitution of even type meningococci is such that a strictly monovalent serum is not produced when horses are injected with a type culture over a long period of time.

The experiments on monovalent sera were made in two ways: first, by injecting as many as three cultures of the same type of meningococcus, and second, by injecting a single type culture only.

Experiment 3.—Horse M 30, after the usual preliminary treatment, was first injected with suspensions of living parameningococci (Cultures 1, 4, and 36). After 6 and 10 months of immunization trial bleedings were made. The serum thus obtained was titrated against 56 stock cultures of meningococci with the results shown in Table III.

TABLE III.

Number of Stock Strains Agglutinated by the Serum of Horse M 30 Immunized with Three Strains of Parameningococcus.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
6	40 of 52	50 of 52
10	56 " 56	56 " 56

The results were striking and unexpected, and yet they confirmed certain tests which we had previously made on the variations in

[8] Hine, T. G. M., *Privy Council, Med. Research Council, Special Rep. Series. No. 50*, 1920, 176.

agglutinogenic activity exhibited by different strains of the meningococcus. It appears that in this respect separate strains of the meningococcus show a wide variation. It has been noted also that horses vary in their response (*cf.* Nos. M 24 and M 25, Table I).

The next step was to determine the effect of immunizing horses with single type strains of the regular meningococcus and the parameningococcus. For this purpose two horses were employed.

Experiment 4.—Horse M 32 was injected with a para culture (No. 1) and Horse M 33 with a regular culture (No. 60). The immunization was begun on December 5, 1918, and test bleedings were made 3 months later at a period still too early to show the final wide range of agglutinative capacities of the sera. However, the serum of Horse M 32 (para) agglutinated in dilutions of 1: 400 or higher 52 of the 56 stock cultures, and the serum of Horse M 33 (regular) agglutinated in the 1: 400 dilution or higher 51 of the 56 stock cultures.

These results raise certain very pertinent questions which cannot be answered offhand. We have dealt with certain aspects of these questions in a later section of this paper in connection with the changes which take place in the sera during storage and the response of the sera produced in different ways to selective absorption tests. We possess many observations which point to the greater efficacy of a truly polyvalent antimeningococcic serum in practice, and hence we do not accept, as yet, such an apparently polyvalent serum, arising from single type cultures, as being immunologically and therapeutically equivalent to the former. The results given show also that an immune horse serum cannot be used for classifying meningococci, and finally, that the so called monovalent sera prepared in France and England in the horse doubtless possessed agglutinative capacities far wider than is implied in their names.

Comparative Agglutinin Content of Monovalent and Polyvalent Sera.

The experiments on horses with single and with several strains of meningococci have shown that agglutinin formation is induced not only for the strain or strains injected but also for a wide number and diversity of other type and subsidiary strains. At first sight it may appear that the sera produced with single and with multiple strains are practically identical and could be substituted for each other in

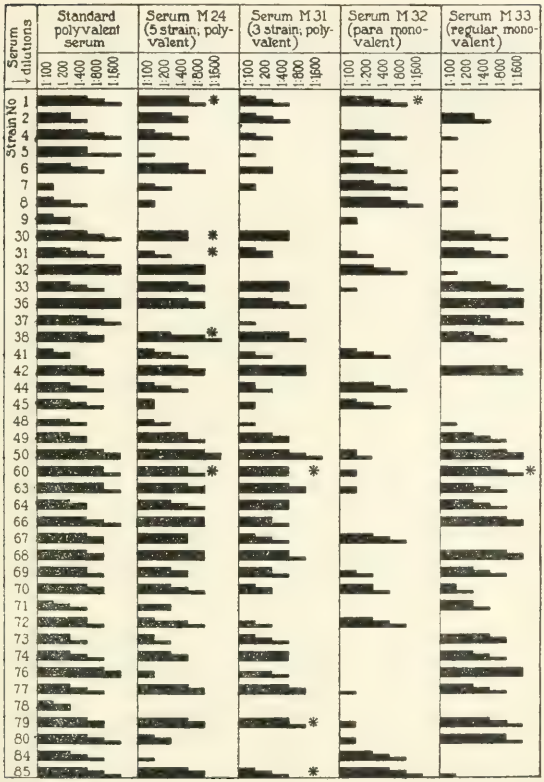
treatment. And yet this deduction would not be justified, as the following tests show. It is also in conflict with observations on the efficacy of widely polyvalent antimeningococcic serum known to contain specific agglutinins for the main and subsidiary strains of the meningococcus.

Effects of Storage.—The first tests to be described here relate to the comparative keeping qualities of the two kinds of sera. The samples of mono- and polyvalent horse sera were kept in the refrigerator in the dark at an approximate temperature of 4°C. for about 1 year. All the sera had been preserved with 0.15 per cent tricresol. The sera tested were derived from Horses M 32 (monovalent para), M 33 (monovalent regular), M 24 (regular, para, and three intermediates), and M 31 (three regular). The polyvalent serum used for comparison was obtained by pooling the serum of six horses, each immunized over a long period of time with 50 odd strains.

The tests of keeping qualities took into account only the agglutinins, which can be quantitatively determined. The titrations were made with suspensions of killed cultures which were uniform for all the tests.

The results of the tests are shown graphically in Text-fig. 1. While the polyvalent serum has fallen off but little during a year's storage and still agglutinates all of the 41 strains of meningococci employed, the two monovalent sera have lost agglutinating power for a considerable number of the strains (sixteen in the case of Horse M 32 (para type) and ten in Horse M 33 (regular type)). On the other hand, the three strain serum (Horse M 31) and especially the five strain (Horse M 24) approach in value the polyvalent serum. The serum of Horse M 24, in a dilution of at least 1: 50, agglutinated 39 of the 41 strains.

The chart brings out the important fact that the common or secondary agglutinins are the first to disappear from the serum and that specific agglutinins for homologous strains are reasonably stable during storage. This is a point of capital distinction and may well prove to be the determining factor in respect to the manufacture as well as to the therapeutic efficacy of the various sera. The striking difference as shown by the pooled polyvalent serum relates to its inclusiveness for essentially all the strains employed in the test. It



TEXT-FIG. 1. An agglutination test with five sera after storage for 1 year or longer in the ice box. In four of the sera asterisks indicate homologous strains.

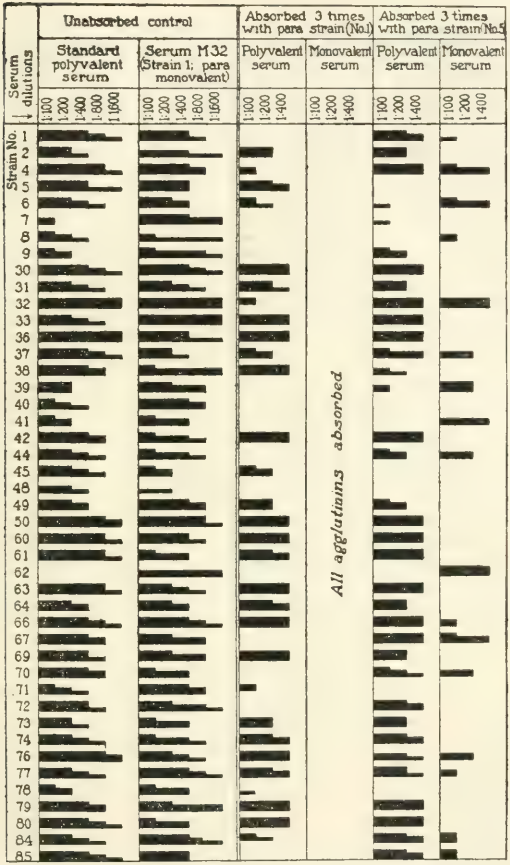
Complete agglutination in any dilution is represented by a broad black band and indicates the flocculation of all organisms, leaving a clear supernatant fluid, even after shaking. Incomplete agglutination, represented by a band of medium width, indicates the flocculation of almost all the organisms, the supernatant fluid remaining hazy after shaking. Partial agglutination, represented by a narrow band, indicates the presence of distinct flocculi which do not disappear on shaking, although the supernatant fluid is cloudy with unagglutinated organisms.

is obvious that as the monovalent sera lose their secondary (or common) agglutinins they become more suitable for use in type diagnosis, and *pari passu*, more unsuitable for therapeutic purposes. One further matter may be pointed out. The standard polyvalent serum was prepared by pooling the sera derived from six horses. The sera prepared with three and five strains respectively came each from one horse. Reasoning by analogy and taking into account the response of different horses to injections of various strains of meningococcus, for example, Horses M 24 and M 25, it is quite possible that the pooling of sera prepared from a small number of type strains might yield an enduring and still more inclusive serum.

Effects on Absorption of Agglutinin.—The differences in deterioration on storage point to a fundamental difference in the monovalent as compared with the polyvalent sera which is confirmed by the test for absorption of agglutinin as is shown graphically in Text-fig. 2.

In Columns 1 and 2 are shown the control agglutination tests made with a 14 month sample of the pooled polyvalent serum and a fresh sample of monovalent para serum (Horse M 32) before absorption with killed cultures of Strains 1 (the homologous para strain) and 5 (also of para type). After triple absorption under carefully controlled conditions, it was found that the single strains had removed all the agglutinins with which they could react, since further absorption did not reduce significantly the agglutinins remaining in the serum.

Text-fig. 2 shows that Strain 1, the homologous strain, exhausted the monovalent serum completely, but was unable to exhaust the polyvalent serum, leaving agglutinins with which 30 of the 44 test strains were able to react. Similarly, while Strain 5, another strain of the same type, removed from the monovalent serum all the agglutinins with which 27 of the 44 test strains had reacted in the control tests, a similar treatment of the polyvalent serum left available agglutinins for all but 9 of the 44 test strains. This experiment emphasizes the difference in the inherent character of the polyvalent and the monovalent serum.



TEXT-FIG. 2. A comparison by agglutination of standard polyvalent serum and of para monovalent serum before and after absorption three times with a heavy suspension of Strain 1 or 5.

Employment of Killed Cultures.

The preparation of therapeutic meningococcic serum with living antigen is laborious and difficult. Because of the rapidity with which the meningococcus dies in artificial cultures, frequent transfers on serum media must be made, and the cultures to be used for the immunizing injections must be freshly prepared each time in a plain agar medium. The question arises, therefore, whether the practical operations cannot be simplified by the employment, at least over certain periods, of killed cultures of the meningococcus so prepared as to prevent the autolysis which tends to destroy the antigenic properties. We have not carried out an exhaustive study of this subject but from many tests on rabbits and the following experiment with Horse M 28, we are of the opinion that the subject is worthy of thorough investigation.

Experiment 5.—Because of the rapidity with which the meningococcus autolyzes in cultures, growths in plain agar in Blake bottles, incubated at 37°C. for 8 to 10 hours, were employed. The surface growths were washed off with 20 cc. of isotonic saline solution and the heavy suspensions were quickly heated in a water bath to 65°C. to destroy the autolytic ferment. Test for viability was made and 0.35 per cent tricresol added. The suspension was kept in the refrigerator.

Horse M 28, after the usual preliminary treatment, was injected, beginning January 4, 1918, with suspensions of killed cultures of the same strains that were used in the immunization of Horses M 24 and M 25; *viz.*, No. 1 (para), No. 60 (regular), and Nos. 30, 31, and 38 (intermediates). The test serum bleedings were made after 7 and 12 months of immunization with the results shown in Table IV.

TABLE IV.

Number of Stock Strains Agglutinated by the Serum of Horse M 28 Immunized with Killed Cultures of Five Strains.

Duration of immunization.	Agglutination test positive.	
	1:400 or higher.	1:100
mos.		
7	46 of 60	59 of 60
12	53 " 56	56 " 56

This single experiment may be taken merely to indicate that as far as the agglutinin response is concerned, killed cultures of me-

ningococci can be used for immunizing horses. It will be patent from all the circumstances and from what has been stated above, that this fact is not regarded as tantamount to the conclusion that a therapeutically active and efficacious antimeningococcic serum can be produced in this way. We should, indeed, be willing to go no further at the present time than to propose that the suspended killed cultures be kept on hand to be used on occasion in place of the suspended live cultures when for any reason circumstances make it impossible to inject the latter. During the war when antimeningococcic serum was being produced at The Rockefeller Institute under great pressure, we never relied on the killed cultures alone and, at most, only occasionally alternated the injection of the killed and the living cultures in the routine manufacture of antimeningococcic serum. We still believe in the practice of employing live cultures and also of cultures freshly isolated from cases of epidemic meningitis.

The power of stored killed cultures, preserved with tricresol, to induce agglutinin formation was tested in one instance in rabbits. The killed cultures had been in the refrigerator for periods of 8 and 14 months. The rabbits tolerated the usual doses. One rabbit injected with a killed regular strain gave a serum of 1: 1,600 titer, and another injected with a killed para strain gave a titer of 1: 800 against freshly prepared killed cultures. The stored killed suspensions were agglutinated in other immune rabbit sera in high dilutions. As a conservative routine, however, the killed cultures employed occasionally for injection and for agglutination were not used after 3 months storage.

SUMMARY AND CONCLUSIONS.

In an attempt to simplify the manufacture of an efficacious antimeningococcus serum an experimental study has been made of a number of sera produced with a few or with single strains of meningococcus, the therapeutic polyvalent serum produced at The Rockefeller Institute with more than 50 strains being used as a standard of comparison.

It was found that horses injected with an antigen limited to five, three or even one strain yielded sera with a range of agglutinins covering in high dilution practically all the stock strains used in pro-

ducing the polyvalent serum. These sera appeared to equal the polyvalent serum in range and titer of agglutinins, but on further examination fundamental differences were found. Storage for a year had little effect upon the titer and inclusiveness of the polyvalent serum, whereas the monovalent serum had fallen off greatly, especially in regard to secondary or subsidiary agglutinins, so that only a comparatively small number of stock strains was still agglutinated. The serum made with five strains, a regular, a para, and three intermediate meningococci, approached the polyvalent serum in keeping qualities and still agglutinated at the end of this period 39 of the 41 strains tested.

Absorption tests also brought out inherent differences in the nature of the polyvalent and the monovalent sera which had appeared to be practically identical in simple agglutination tests. The homologous strain on triple absorption was able to exhaust the monovalent serum completely, but was unable to remove from the polyvalent serum agglutinins to which 30 of 44 different strains were able to react. Absorption with another single strain of the same type removed from the monovalent serum agglutinins for a majority of the test strains but left the polyvalent serum relatively unaffected.

It is comparatively easy to produce a serum effective against about 80 per cent of the spinal strains of meningococci encountered. Deficiencies in our knowledge of the antigenic capacities of the meningococcus have led to the more or less empirical use of a large number of cultures in the preparation of a serum effective against the remaining 20 per cent of the strains. How far the number of the latter in the antigen may be reduced without restricting the efficacy of the serum remains yet to be determined. However, the experimental evidence recorded here apparently does not favor the use of an antigen limited to one or too few strains. For example, three or five selected strains produced a serum which agglutinated practically all the strains against which it was tested. But in view of the many observations which point to the greater therapeutic efficacy of a serum made with a larger number of strains we would not as yet advocate a serum prepared with too limited antigens even though it contains at first a wide range of agglutinins.

It has been brought out that a monovalent serum contains, in addition to specific agglutinins, a wide range of common or secondary agglutinins which tend to disappear during storage. The difference between specific and secondary agglutinins is not apparent in simple agglutination tests, but is revealed by absorption tests. It is probable that in a serum prepared with a few strains the same condition exists, whereas in a serum produced with a large number of strains the agglutinins are mainly specific as contrasted with the fact that most of them are secondary in the serum produced with few strains. The question whether secondary agglutinins are therapeutically equivalent to primary or specific agglutinins requires further study.

We wish to acknowledge our indebtedness to Dr. J. H. Brown and Dr. R. B. Little, of the Department of Animal Pathology of The Rockefeller Institute, for their cooperation.

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THE RESISTANCE (OR IMMUNITY) DEVELOPED BY THE REACTION TO SYPHILITIC INFECTION.

And Some of the Effects of the Suppression of this Reaction.

By WADE H. BROWN, M.D., and LOUISE PEARCE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The idea of an inhibitory influence has been suggested as a means of explaining certain phenomena of syphilitic infections and there is some experimental evidence on which to base such a conception. The difficulty has been that most of the evidence cited in support of this theory was susceptible of more than one interpretation. This applies in particular to reinoculation and superinfection experiments which have been the favorite means of demonstrating inhibitory effects. Nowhere, however, is the action of such an influence more strongly suggested than in the experimental infection produced in the rabbit by scrotal or testicular inoculations of well adapted strains of *Spirochaeta pallida*.

As it is ordinarily seen, the two most striking features of this infection are an extremely marked reaction at the site of inoculation and a total absence of generalized manifestations of disease. If one considers these peculiarities of the experimental infection as compared with that in man, one can hardly fail to see a possible connection between the two conditions mentioned, especially when it has been shown that the absence of generalized lesions in the rabbit cannot be accounted for either by a lack of dissemination of spirochetes or by a tissue insusceptibility.

It appeared to us that the reaction to infection as it occurred in the rabbit offered an opportunity for determining the influence which a syphilitic reaction in one part of the body might exert on manifestations of the disease elsewhere and, at the same time, a possibility of modifying the experimental infection so as to increase its usefulness as a means of investigating problems of human syphilis.

Accordingly, a series of experiments was carried out with a view to determining the effect which a simple reduction or suppression of the reaction at the site of inoculation might have on other clinical manifestations of the infection.

EXPERIMENTAL.

The means employed in making these comparisons were unilateral as contrasted with bilateral inoculation, unilateral and bilateral castration at various stages of the infection, suppression of the primary reaction by the use of therapeutic agents and complete prevention of a primary reaction. (Castrations were made under ether anesthesia.)

The effects produced were gaged on the basis of the occurrence of generalized lesions. In the rabbit, the conditions which one may recognize clinically include various forms of cutaneous lesions, lesions of the mucous membranes, lesions of the periosteum and bones, lesions of the eyes and a general lymphadenitis. The latter condition is of such common occurrence, even when no other clinical sign of generalized infection can be recognized, that it was not used as a standard of comparison.

The results obtained from these experiments will not be reported in detail, but the essential character of the effects produced by the procedures indicated will be given briefly.

Effects of Unilateral and Bilateral Inoculation and of Castration.

In the first group of experiments, a comparison was made of the effects of unilateral and bilateral inoculation and of the removal of the infected testicles soon after the appearance of the initial lesions. The results of these procedures were determined on the basis of a period of four months' observation.

Bilateral Inoculations.—Of twenty rabbits inoculated in both testicles, fourteen were castrated and six were held as controls. Generalized lesions developed in one of the six controls and in thirteen of the fourteen castrated animals within the period of observation.

Unilateral Inoculations.—Twenty-seven rabbits were inoculated in one testicle only; fourteen of these were castrated and thirteen were

held as controls. In this series, generalized lesions developed in eight of the thirteen controls as contrasted with one of six animals inoculated in both testicles and again in thirteen of the fourteen castrated animals.

Several other experiments of a similar character gave esssentially the same results, thus showing that by inoculating one testicle instead of two, the incidence of generalized lesions was markedly increased. When the reaction at the site of inoculation was further reduced by early removal of the infected organs, generalized lesions developed in almost every instance.

Effects of Suppression of Primary Lesions by Therapeutic Agents.

In a second set of experiments in which castrations were made at different stages of the infection, the effects of suppression of the testicular lesion by the use of a therapeutic agent was also tested. For this purpose, a drug was chosen from among those studied by us in collaboration with Dr. W. A. Jacobs and Dr. Michael Heidelberger, whose effect in inducing resolution of syphilitic lesions was much greater than its spirocheticidal action. This substance was arsenophenylglycyl dichloro-m-aminophenol.

Twelve rabbits, six of them inoculated unilaterally and six bilaterally, were given a single intravenous injection of this drug (5 mg. per kilo) fourteen days after inoculation, and the results were controlled by six untreated rabbits from each of the respective groups.

In the unilateral series, the lesions present were almost completely resolved and the local reaction suppressed for between two and three weeks. At the end of three months, all of these animals had developed generalized lesions as contrasted with three of the six controls. The effect of the drug on the animals inoculated in both testicles was less marked and lasted for only from seven to ten days. At the end of three months, generalized lesions had developed in four of the six treated animals and in one of five surviving controls.

This experiment showed that by properly gaging the dose of a therapeutic agent so as to suppress the lesions present without destroying the infecting organisms, the infection can be intensified in the same way as by an excision of the primary lesions.

Effects of Complete Prevention of a Reaction at the Site of Inoculation.

In another experiment, the reduction of the reaction at the site of inoculation was carried to the point of complete prevention. This was accomplished by inoculating ten rabbits in the right scrotum, using tissue implants, and at the end of forty-eight hours completely excising the scrotum and testicle of that side under ether anesthesia.

By the end of the seventh week, eight of the ten rabbits in this series showed a marked generalized syphilis and the other two developed slight generalized lesions at the end of two and two and one-half months.

Taken as a whole, the generalized infection in this series of animals was the most pronounced which we have seen in any single group of rabbits. This would indicate that both the incidence and severity of the generalized infection tend to increase in proportion to the reduction or suppression of the reaction at the site of inoculation.

CONCLUSION.

These experiments show that, in so far as syphilitic infections in the rabbit are concerned, the reaction which takes place at the site of inoculation tends to dominate the entire course of the infection; that, in effect, this reaction either inhibits or obviates the necessity for the development of lesions elsewhere and, conversely, that the reduction or suppression of the reaction by the use of any means that does not exercise an equal effect on the organisms themselves, removes this control and tends to increase the occurrence of generalized lesions and the severity of the infection.

If one is prepared to accept the infection produced in the rabbit by *Spirochaeta pallida* and the reaction to infection on the part of the experimental animal as analogous in kind to those in man, these observations become of far reaching importance and may open the way to a better understanding of many problems of human syphilis.

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LOCAL AUTOINOCULATION OF THE SENSITIZED ORGANISM WITH FOREIGN PROTEIN AS A CAUSE OF ABNORMAL REACTIONS.*

By JOHN AUER, M.D.

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Plates 45 and 46.

(Received for publication, June 9, 1920.)

INTRODUCTION.

While testing the sensitiveness of a number of dogs which had been treated with horse serum some years previously, and employing heavy doses of horse serum for the reinjections, it was observed that a peculiar edema developed at the site of the operation wound in the inguinal region. This edema was noted about 2 days after the test and formed a fairly extensive, thick, brawny mass of tissue; there was no discharge from the wound. In order to explain this peculiar type of edema, I assumed that the local reaction was anaphylactic and was produced in the following fashion. In these dogs a foreign protein (horse serum) was circulating, due to the reinjection, and therefore a certain amount of this protein ought to pass into the tissues adjoining the wound during the development of the ordinary wound edema which always follows an operation. Moreover, the amount of foreign protein acting locally would be increased by the oozing of blood, serum, plasma, and lymph into the wound from the severed blood and lymphatic capillary channels, all of which contain the antigen. As the dogs were sensitized to this foreign protein, the skin and adjoining tissues would also be sensitized and could respond by an anaphylactic reaction to this local autoinoculation of the horse serum.

Since this working hypothesis of local autoinoculation could readily be utilized to explain functional changes in any tissue capable of react-

* A preliminary note appeared in the *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 93.

ing anaphylactically, and since its validity could easily be tested experimentally, and since in addition the conception was new, as far as I was aware, a number of series of experiments were carried out to determine its viability.

EXPERIMENTAL.

Method.

The first tests were made in dogs which were sensitized by the subcutaneous injection of horse serum and then reinjected intravenously after an interval of 4 weeks. At the time of reinjection a deep incision through the skin was made under ether anesthesia with antiseptic precautions, then sutured, bandaged, and the process of healing observed. Although the results were encouraging, it early became evident that practical considerations made the dog an unsuitable test object. For this reason the rabbit was finally selected, and after preliminary tests the skin was again chosen as indicator organ of the reaction. The specific site was the ear, because this could be examined with the maximum of ease and the minimum of discomfort for the animal.

The rabbits were sensitized by four injections of 4 cc. of horse serum at 4 to 5 day intervals. Two injections were given into the erector spinæ muscles and two into the peritoneal cavity. After an incubation period of 15 to 21 days they were reinjected with 10 cc. of horse serum, usually intraperitoneally. In one series half the reinjection dose was given into the erector spinæ muscles and half into the peritoneal cavity. The intravenous route for reinjection was not employed because a test showed a large number of fatalities in the sensitized group. Thus in Series 2 four out of six sensitized rabbits died in 2 to 15 minutes after the intravenous injection of 5 cc. of horse serum, which made this series valueless.

The initial local damage to provoke a local edema and inflammation was produced by painting the external surface of the ear with 1 cc. of xylol and rubbing it gently for 15, 20, or 30 seconds, the time interval depending upon the series. This skin irritant causes a marked temporary flushing of the ear vessels which is followed by a mild inflammation and edema of varying degree. It will be noticed that this skin irritant was used to bring about the same condition as the skin incision in dogs, the production of a certain degree of inflammation and exudate into the tissues. In Series 1 a chemically pure *o*-xylol (Kahlbaum)[1] was used; in the others a commercial xylol (Merck) was employed.

Commercial xylol was chosen for the later series because the chemically pure isomers were available only in small amounts and also because the commercial

[1] I am indebted to Dr. W. A. Jacobs for supplying me with some of Kahlbaum's *o*-, *m*-, and *p*-xylol. It may be mentioned that *o*-xylol is more effective in producing edema than *m*- or *p*-xylol.

product seemed to be more effective in producing a local edema. Since the commercial product varies in its percentage composition of *o*-, *m*-, and *p*-xylol, and since it probably also contains various impurities, commercial xylol (Merck) from the same container was used throughout the subsequent series.

The same dose of 1 cc. of xylol was given to all rabbits and was always measured by the same pipette.

The xylol was usually applied to the skin 30 to 45 minutes after the reinjection of horse serum; in one series the interval was 3 hours. These intervals were allowed to pass in order to permit the absorption of a certain amount of horse serum, so that any edema developing after the xylol application would contain some of it.

The rubbing of the ear was always gentle and was done with a finger covered by a rubber cot. As the ear had to be held during the rubbing, some of the xylol always moistened the finger on the inner surface of the ear. To a certain extent, therefore, xylol was applied to the outer and inner ear surfaces, though by far the most was received by the external surface. The time during which the ear was rubbed was always controlled by a clock (15, 20, or 30 seconds, depending upon the series).

For each sensitized and reinjected rabbit at least two different control experiments were carried out at the same time. In one series of controls the normal rabbits received 10 cc. of horse serum intraperitoneally 30 to 45 minutes before treating the ear with xylol (serum controls). In the second series of controls no horse serum was administered, and the only interference was painting the ear with xylol (ordinary controls). In a third group of controls the rabbits were sensitized with horse serum, and after the incubation period they were not reinjected, but xylol was applied to the ear (sensitized controls). In general each series of experiments consisted of 18 rabbits: 6 sensitized and reinjected; 6 horse serum controls; and 6 normal controls.

The horse serum[2] employed was always at least 4 months old. In the earlier series the serum was sterile and without any preservative; in later series the serum contained chloroform. When chloroform was present this was driven off by floating a sterile evaporating dish containing the serum in a dish of warm water. As far as possible the same lot of serum was used for sensitizing and reinjection.

The injections were always made with a sterile syringe, and the site of puncture was prepared by cutting the hair and treating the area with dilute tincture of iodine.

All the rabbits were examined first at 2 to 4 hour intervals; then daily; later at 2 to 3 day intervals. The entire period of examination lasted about 2 weeks when no reaction was obtained; if a reaction took place the examinations were usually continued for 3 or 4 weeks.

Most of the rabbits employed were males; when females had to be used the same number of females was added to each group in a series. All were kept in

[2] The horse serum was obtained from the New York City Board of Health through the kindness of Dr. W. H. Park, Dr. E. J. Banzhaf, and Dr. I. Greenwald.

individual cages. The weight fluctuated between 1,400 and 2,000 gm. The diet consisted of oats, hay, cabbage, and turnips; occasionally carrots were available. The ordinary gray variety formed the majority; no white rabbits were used because they could not be obtained in sufficient numbers. As far as possible young adult animals were employed; very young rabbits were never used.

Results.

Before describing the experimental results more fully, it may be stated at once that the work strongly supports the working hypothesis upon which the investigation is based. Thus in three series of experiments, totalling 53 animals, the xylol-treated ears of 36 controls showed no dermatitis with blisters and crust formation; nor were hemorrhages and gangrene observed except in one and two instances respectively. In the latter instances the loss of substance did not exceed 0.5 mm. of the ear tip. The ears of these controls were practically normal 3 to 4 days after the xylol application.

The picture was, however, quite different in the sensitized and reinjected groups. Out of seventeen rabbits, ten developed an exfoliative dermatitis a few days after the xylol treatment. The blisters and crusts covered one-third to one-half of the ear surfaces, involved the deeper tissues, and always led to dry gangrene in these particular animals. The gangrene caused a loss of ear substance at the tip, varying from 1 to 3 cm. Healing was slow, but usually complete in 3 to 4 weeks. The ear stumps were at first bald, with a glistening, thin skin; later a new growth of white hair appeared (Figs. 1 to 3).

The ear changes may now be considered more in detail. When 1 cc. of *o*-xylol or commercial xylol is applied to the hairy surface of the rabbit's ear and then gently rubbed for 15 or 30 seconds, a marked dilatation of the blood vessels is usually produced fairly promptly. This flushing of the ear lasts longer than 10 minutes, but has usually disappeared after 45 minutes. At this time the ear generally presents a faint pink flush and the vessels are narrow. After 3 to 6 hours a fair to moderate edema appears in a majority, but not in all of the treated ears in the two control groups. Within 48 hours the edema in these two groups has practically disappeared in most animals, and in 3 days the treated ears are largely normal, if we exclude a slight desquamation, a slow loss of hair which is promptly replaced by normally pig-

mented hair, and a tendency of the ear arteries to flush more readily and more strongly than in the untreated ear (Table I).

TABLE I.

Onset and Course of Edema in Series 1, 3, and 4.

Time.	Sensitized and reinjected rabbits (17).	Horse serum controls (18 rabbits).	Ordinary controls (18 rabbits).
hrs.			
3–6	No or slight edema, 14. Slight to fair edema, 3.	No or slight edema, 5. Fair to moderate edema, 13.	No or slight edema, 7. Fair to moderate edema, 11.
22–27	No or slight edema, 10. Fair to moderate edema, 1. Moderate to marked edema, 6.	No or slight edema, 11. Fair to moderate edema, 6 (1 died).	Slight edema, 16. Moderate " 1. Marked " 1.
48	No or slight edema, 11. Fair to moderate edema, 3. Marked edema, 3.	No or slight edema, 14. Fair edema, 2. Moderate edema, 1.	No or slight edema, 16. Fair edema, 1. Moderate edema, 1.
72	No or slight edema, 11. Moderate edema, 2. Marked edema, 3 (1 died).	No or slight edema, 16. Slight to fair edema, 1.	No or slight edema, 16. Fair edema, 2.
120	No or slight edema, 13. Marked edema, 3.	No or slight edema, 14. Marked edema,* 3.	No or slight edema, 18.

* This recurrent edema practically disappeared the next day and did not reoccur. See text for details.

When the xylol edema has disappeared there is usually no recurrence of it. Only three exceptions were noted in the large number of controls, and they occurred in the horse serum control group of Series 1. These three rabbits had received horse serum for the first time about 45 minutes before the ear had been treated with *o*-xylol (Kahlbaum). A fair edema developed and disappeared after 48 hours. On the 3rd day none of them showed any edema (Table I). On the 5th day (no observation made on the 4th) all three showed a strong edema which was more marked than that seen previously. The next day (6th), however, the edema had practically disappeared in all, and no further edema was noted during the next 9 days, when observations were discontinued. No exudate ac-

companied this recurrent edema, nor did any gangrene take place. One of these rabbits also showed numerous small discrete hemorrhages scattered on the central portion of the upper dorsal half of the ear.

No blisters or crusts were observed in the great majority of both control groups. In the large number of controls only two cases were seen. These two animals belonged to the ordinary controls of Group 3 and had therefore never been subjected to any serum action. The exudate which they showed was very slight, occupied the extreme tip of the ear, and the subsequent dry gangrene of the

TABLE II.

Dry Gangrene of the Ear Tip.

Series No.	Sensitized and reinjected rabbits.	Horse serum controls.	Ordinary controls.
Series 1 (*o*-xylol).	Gangrene, 4.* No gangrene, 2.	Gangrene, 0. No gangrene, 6.	Gangrene, 0. No gangrene, 6.
Series 3† (commercial xylol).	Gangrene, 3. No gangrene, 2.	Gangrene, 0. No gangrene, 6.	Gangrene, 2.‡ No gangrene, 4.
Series 4 (commercial xylol).	Gangrene, 3. No gangrene, 3.	Gangrene, 0. No gangrene, 5 (1 died).	Gangrene, 0. No gangrene, 6.

* The loss of substance in all three groups varied from 10 to 30 mm.

† Series 2 was lost through death of most of the sensitized group due to intravenous reinjection.

‡ The loss of substance was less than 1 mm. of the ear tip.

tip was less than 1 mm. (Table II). The bald tip of these ears later grew a small tuft of unpigmented, white hair.

In the sensitized and reinjected series of rabbits the application of xylol to the ear produces in general the same immediate effects as those noted for the control group, except that the flushing of the ear vessels at times is not quite so prompt in the animals tested 30 to 45 minutes after the horse serum injection; in Series 5, however, in which 3 hours passed before the xylol was applied to the ear, all ten rabbits showed a prompt and strong initial vasodilatation. A similar prompt initial flushing after xylol was also obtained in the group of four

animals which were sensitized but not reinjected. The development of the pink diffuse blush which preceded the appearance of a definite edema was delayed in the reinjected series as well as in the horse serum controls of Series 3 and 4; in Series 1, however, all the rabbits, both the reinjected as well as the two control groups, showed a diffuse pinkness $2\frac{1}{2}$ hours after the xylol application.

A difference between the reinjected groups and the control groups of all the series was shown in the development, degree, and persistence of the edema called forth by the xylol. Table I summarizes these variations. From this table it will be seen that the edema in some rabbits of the sensitized reinjected groups developed more slowly, persisted longer, and reached a greater degree than in either of the control groups. The table also indicates that a somewhat larger percentage of the sensitized reinjected group apparently never passed beyond a slight degree of edema than in either of the control groups.

The most striking difference, however, between the sensitized reinjected groups and the control groups was exhibited in the development of blisters, crusts, and dry gangrene in the xylol-treated ears (Figs. 1, 2, and 3). These lesions occurred practically only in the sensitized reinjected groups, and in them ten out of seventeen rabbits showed extensive lesions. In the control groups, on the other hand, only two rabbits out of thirty-five (serum controls + ordinary controls) showed a slight crust formation at the ear tip which was followed by a minimal loss of tissue (Table II).

The blisters usually were observed in 24 to 48 hours; in one instance only was a bleb seen 6 hours after the xylol application. In typical cases the blisters appear first on the internal surface of the ear along the medial or lateral border. On the external surface usually a dry brownish black crust is first seen. The blisters appear in crops, varying in size from 2 to 4 mm. in diameter; in one instance a single large, thick walled blister 20 by 12 mm. occurred at the tip of an ear. The walls of the blisters are usually fairly thin and the blisters are at first filled with a yellowish fluid which later turns brown and forms a brownish black crust on drying. Some of the blisters also contained blood. In some instances no blisters were seen but exudate and soft crusts were the first alteration noticed. Thus extensive soft crusts were

occasionally seen for the first time 5 days after the xylol application. These crusts may be extensive and approximately one-half of the ear may be covered with them. Along the borders of the ear the crusts always extend farther towards the root than along the middle of the ear. Removal of some crusts showed that the entire skin was involved, for a raw bleeding surface was left exposed (Fig. 2).

Within 3 to 5 days the upper portion of the ear began to fold and curl slightly, and in 7 to 10 days the tip of the ear was black, dry, and hard. Separation of the gangrenous portion took place slowly and usually occurred 11 to 14 days after the xylol application, at which time the process was hastened mechanically. The loss of substance varied in the three series under discussion from 10 to 30 mm. of the tip of the ear (Figs. 1, 2, and 3). The loss of substance along the lateral border was well shown by the fact that the marginal ear vein formed part of the gangrenous area or now appeared within a millimeter or less of the edge of the ear for a portion of its course.

Removal of the dry gangrenous tip and crusts left the upper ear practically bald; the skin was thin and glistening with a few raw and slightly bleeding surfaces; the borders were thickened, nodular, with some tongue-like projections. The entire upper ear was moderately edematous, of a diffuse bluish pink color, and the vessels were moderately blurred on transillumination, probably due to the edema of the surrounding tissues. Healing was usually complete in 3 to 4 weeks.

After 2 weeks the surface of the ear stump gradually became covered with hair, but the new growth of hair was entirely devoid of pigment. This white hair has now persisted without any change for more than 5 months in two typical cases which were allowed to survive.

No effort was made to follow the histological changes of the ear lesions at various stages of their development because this would have entailed the loss of too many animals and at present it was considered more important to observe the gross changes.

One more difference remains to be noted, the occurrence of numerous small discrete hemorrhages on the dorsal surface of the xylol-treated ears of sensitized reinjected rabbits. The fact that the presence of numerous small hemorrhages is being considered should be emphasized at once, for the appearance of an occasional small hemorrhagic spot or two is not infrequent in the ears of apparently normal rabbits. The petechial hemorrhages under discussion were most frequent

in the sensitized reinjected rabbits of Series 1 in which four out of six rabbits exhibited them. Among the twelve controls of this series only one animal, a serum control, developed similar hemorrhages. In Series 3 only one animal showed these hemorrhages, and it was a member of the sensitized reinjected group; the twelve controls showed no hemorrhages. In Series 4 only two rabbits developed petechiæ of the ear, and they again belonged to the sensitized reinjected group; in this series also the twelve controls showed no hemorrhages. To sum up, therefore, petechial hemorrhages occurred in seven out of seventeen sensitized and reinjected rabbits, but only once in thirty-six controls; they were especially frequent in Series 1 (four out of six) and infrequent in Series 3 and 4 (one or two out of six). This difference in frequency of occurrence in the three series is possibly related to the fact that in Series 1 chemically pure *o*-xylol (Kahlbaum) was employed, while in Series 3 and 4 commercial xylol (Merck) was used.

Supplementary Experiments.

Three other series of experiments were carried out for reasons which will be considered later. In the first of these, Series 5, the effect of allowing 3 hours to elapse between the injection of horse serum and the application of xylol was investigated. This series was composed of ten rabbits all of which had been sensitized, but not to the same degree. The number of sensitizing doses given were as follows: 6 injections, 2 rabbits; 2 injections, 3 rabbits; and 1 injection, 5 rabbits. All these animals had been used previously; two had been members of a sensitized reinjected group and the other eight had served as serum controls. 21 days had passed since the last serum injection and the last xylol application. On the day of the test each rabbit received 10 cc. of horse serum intraperitoneally; after 3 hours, none of them having shown any signs of collapse, the left ear was treated with 1 cc. of commercial xylol and gently rubbed for 15 seconds. It should be added that the left ear had not been subjected to the action of xylol in previous tests.

Since these animals form three groups of varying degree of sensitization, it is perhaps of little value to compare them in detail with the series previously described, which again have another degree of sensitiveness. The main point is that one rabbit in each set, that is, three rabbits in all, developed a marked exudate and crust formation on the xylol-treated ear. These crusts were first noted in one rabbit on the 4th day and in the other two rabbits on the 7th day (no obser-

vations unfortunately were made between the 4th and 7th days). The crusts involved both ear surfaces, varied in extent from 2 to 5 cm., and were in all respects apparently similar to those observed in Series 1, 3, and 4. The resultant gangrene, however, was much less than that observed in the earlier experiments; one rabbit lost 6 mm. of the ear tip, and the other two suffered but a slight loss of ear tissue. In the last two cases the bald ear tip was bluish, puckered, and considerably thickened, in one instance to 3 mm. About 3 weeks after the application of the xylol, and 2 weeks after the removal of the crusts, the bald ear tips showed a new growth of white hair.

For the sake of completeness it may be added that after 6 hours the edema produced by the xylol application was only slight in seven rabbits and between slight and fair in three. In the latter three rabbits, the ear later developed blisters, exudate, and crusts as described above. After 48 hours the edema had disappeared in practically all. These rabbits, therefore, as far as delayed onset of the edema and development of a severe dermatitis were concerned, resemble the sensitized reinjected groups of previous series; they differ, however, from the latter in the relatively short duration of the edema which was produced.

The same animals, with the exception of one which was killed, were again used 22 days later in order to test the effect of xylol on the ears of sensitized but not reinjected rabbits. The rabbits therefore were only subjected to the action of 1 cc. of commercial xylol applied to the same ear which had served in the previous test; the ear was then gently rubbed for 15 seconds. An edema was noticed after 2 hours which increased moderately after 6 hours and practically disappeared after 24 hours. No blisters, crusts, hemorrhages, or gangrene of any degree were observed during the next 13 days, at the end of which time the animals were discarded. It will be observed that this group of sensitized but not reinjected rabbits behaved like ordinary normal control rabbits.

In order to verify still further the action of xylol upon sensitized but not reinjected rabbits, five normal animals were each sensitized by four subcutaneous injections of 1 cc. of horse serum, at 3 to 6 day intervals. When 21 days had elapsed after the last serum injection the left ears of the four survivors[3] were treated with 1 cc. of com-

[3] One rabbit died during the process of sensitization.

mercial xylol, and then lightly rubbed for 15 seconds. All the ears flushed promptly and strongly on application of the xylol; after $1\frac{3}{4}$ hours all showed a fair to moderate edema which increased up to 6 hours after the xylol treatment. 22 hours afterwards the edema in all had decreased, and in 48 hours it had practically disappeared. These rabbits were examined daily for 14 days and at no time were any hemorrhages, blisters, or crusts seen. In this group again the sensitized but not reinjected rabbits had behaved like the ordinary control groups.

DISCUSSION.

It has been clearly demonstrated above that a mild skin irritant, such as xylol, produces strikingly different effects when applied to the ears of control rabbits or to the ears of rabbits previously sensitized and reinjected with horse serum. In normal as well as in serum control animals or rabbits which have been sensitized but not reinjected, the xylol produces only a moderate inflammation with edema, and no blistering or obvious gangrene results. In sensitized and reinjected rabbits, on the other hand, xylol exhibits in a majority of the rabbits a marked blistering effect, followed by heavy crust formation and tissue destruction; the tissue destruction often leads to a dry gangrene involving several centimeters of the ear tip. It is thus seen that xylol acts like a rubefacient in the three types of controls, while in the sensitized and reinjected rabbits the same agent, applied in the same dose and in the same way, behaves like a vesicant and escharotic.

In an interpretation of these findings the most obvious, indeed the only explanation is that an anaphylactic reaction plays a dominant part, since the lesions were observed in sensitized and reinjected rabbits only. The ear lesions, therefore, may be the direct expression of a local anaphylactic reaction, as the working hypothesis postulates, or they may be the secondary result of an anaphylactic reaction or reactions taking place elsewhere than in the ears.

In an analysis of the view that the lesions are the secondary result of anaphylactic reactions the general reactions which come into consideration are (1) an anaphylactic fall of general blood pressure; (2) local changes in the ear circulation due to general anaphylactic reaction; (3) anaphylactic abnormalities of cardiac action; and (4) anaphylactic changes in the blood.

Fall of Blood Pressure.[4]—Though no blood pressure determinations were made for obvious reasons, it can be definitely stated that a profound drop did not occur. This is demonstrated by the fact that none of the sensitized rabbits showed a definite prostration or collapse after the reinjection, a condition which always accompanies a severe lowering of the blood pressure in anaphylactic rabbits. If a fall of blood pressure occurred, it therefore must have been at most a moderate drop. Concerning the duration of this hypothetical blood pressure fall, indirect evidence must be sought from experiments in which the reinjected dose was given intravenously, for there is no record of blood pressure studies in the anaphylactic rabbit when the reinjection is carried out by way of the peritoneal cavity, which is the route employed in the experiments reported in this paper. Now it may be justly assumed that the blood pressure fall of sensitized rabbits is surely not severer or of longer duration in rabbits reinjected peritoneally than in similarly sensitized rabbits in which the intoxicating dose is administered intravenously. I have numerous instances of the latter type from earlier experiments,

TABLE III.

Blood Pressure Fall of Sensitized Rabbits Reinjected Intravenously.

	Normal blood pressure.	Lowest level after injection.	Recovery level.	Time required for recovery level.	Dose of serum.
	mm.	*mm.*	*mm.*	*min.*	*cc.*
1	110	56	103	20	10
2	104	63	103	30	—
3	118	80	110	12	10
4	116	51	108	25	10
5	130	85	108	17	20
6	120	79	110	6	10

and Table III illustrates the degree of blood pressure fall and the time required to regain a practically normal level. These rabbits were sensitized by repeated (spaced) injections of horse serum at intervals, and were reinjected intravenously with 10 cc. of the same serum. The blood pressure was recorded with a Hürthle membrane manometer. Many mates of these animals succumbed acutely to the same reinjection dose.

Table III illustrates how promptly, within 6 to 30 minutes, well sensitized rabbits may regain a practically normal blood pressure level even though the anaphylactic blood pressure drop is severe.

It must be remembered that the xylol was never applied to an ear until at least 30 to 45 minutes had elapsed after the intraperitoneal injection of 10 cc. of horse serum; in one series (No. 5) 3 hours were allowed to pass. It may then be concluded with a fair degree of certainty that all the animals had recovered

[4] Arthus, M., *Arch. internat. physiol.*, 1908–09, vii, 479.

largely if not entirely from the anaphylactic fall of blood pressure before the xylol was applied to the ear. The anaphylactic fall of blood pressure, therefore, may be disregarded as a factor in the production of the ear lesions.

Local Changes in the Ear Circulation.—A factor of perhaps greater importance than a fall in the general blood pressure is the degree of circulation which is maintained in the xylol-treated ear. The circulation in the treated ears of the sensitized reinjected rabbits might be less efficient than in the xylol-treated ears of controls. This factor was controlled as far as possible by inspection at $2\frac{1}{2}$, 3, and 6 hour intervals after the application of the irritant. There was no significant change in the vascularity of a majority of the ears in the sensitized reinjected groups which was not also present in a majority of one or both control groups at the same time. One factor perhaps indicates that the circulation in the xylol-treated ears of the sensitized reinjected group was not so good as in the control ears; *viz.*, the fact that the edema developed more slowly than in the control group. It is also possible, however, that this delay in the onset of edema is the result of blood changes which will be touched upon later. On the whole, however, there is no definite evidence that the circulation is poorer in the sensitized reinjected group of rabbits after the application of xylol than in the control ear.

Abnormalities of Cardiac Action.[5]—These are very frequent in anaphylactic rabbits after intravenous injections but their duration is relatively short. As a rule they last only 7 to 21 minutes after the reinjection. Consequently, even if it is granted that the same cardiac changes appear in sensitized animals after intraperitoneal reinjection, the animals had ample time to recover before the xylol test was made (30 to 45 minutes and 3 hours after the serum injection).

Anaphylactic Changes in the Blood.[6]—What effect variations in the coagulability of the blood, alterations in the blood picture, and other anaphylactic hemic changes have upon the resistance of body cells is not known, and their influence in the production of the xylol ear lesions cannot therefore be estimated. As mentioned before, these factors may perhaps be the cause of the delayed onset of the edema in the sensitized reinjected animals.

An observation which deserves mention is that the xylol-treated ears of the sensitized and reinjected group often remained cooler than the non-treated ear of the same animal for some hours; after this period they became warmer than the non-treated ear. The treated ears of the control groups, on the other hand, usually were warmer than the non-treated ears within 2 hours after the application of the xylol. There was, however, no relation between the incidence of blisters, skin destruction, and gangrene and this initial coolness of the ear; the lesions

[5] Auer, J., and Robinson, G. C., *J. Exp. Med.*, 1913, xviii, 450.

[6] Arthus, M., *Arch. internat. physiol.*, 1910, ix, 157. von Pirquet, C., and Schick, B., Die Serumkrankheit, Leipsic and Vienna, 1905. Schlecht, H., and Schwenker, G., *Deutsch. Arch. klin. Med.*, 1912, cviii, 405. Weinberg, M., and Séguin, P., *Compt. rend. Soc. biol.*, 1914, lxxvi, 585.

occurred and failed to occur in ears which had been cooler than the untreated ears for the first few hours; similarly they appeared or failed to appear also on ears which had been warmer than the untreated ear of the same animal. There was apparently, however, a definite relation between the initial coolness of the treated ear in the sensitized reinjected group and the subsequent degree of edema; if the ear was initially cool for some time, the edema which developed later (before the onset of skin inflammation) was generally slight. From the facts stated above it follows that the degree of edema bears no relation to the incidence of ear lesions. Typical lesions of the treated ear in the sensitized reinjected groups occurred after slight, as well as after marked edema.

From this survey it may be concluded that none of the factors discussed offers a well founded, satisfactory explanation of the ear lesions; in spite of this, however, it is possible that some, or perhaps even all may play a part in the circle of events which are necessary to produce the end-result.

We come now to the alternative explanation, that the ear lesions are the expression of a primary, local, anaphylactic reaction. This interpretation is the one upon which the investigation was founded and which was briefly stated in the beginning of this paper. The working hypothesis assumed that a sensitized organism, under certain conditions, could reinject itself locally with the same antigen which caused sensitization, thus producing the state necessary for an anaphylactic reaction in that locality. The conditions were (*a*) the presence of the antigen in the circulating juices of the sensitized organism, and (*b*) the local accumulation of this antigen in a tissue showing some degree of inflammation.

These conditions were met experimentally by employing rabbits sensitized with horse serum, and, after an incubation period, reinjecting them intraperitoneally with a readily tolerated dose of the same serum. After a time the blood and lymph would necessarily contain some of the horse serum due to absorption from the injection depot. The local accumulation of the antigen in an inflamed area (condition *b*) was brought about by the application of a mild skin irritant, xylol, upon the ears of the rabbits. As this irritant causes inflammation and edema of the ear tissues, it is obvious that the edema fluid would contain more or less of the horse serum, for the material composing the edema must necessarily come from the blood and lymph capillaries in which this antigen is circulating and whose permeability has

been increased by the inflammatory process. A reaction between the sensitized inflamed tissue cells and the antigen could now take place even though the concentration of the horse serum were not greater in the inflamed and edematous area than in the lymph spaces of non-inflamed tissues of the same animal. The reason is that in the inflamed tissues the metabolism of the cells is greatly increased, and we may therefore justly assume that a larger amount of antigen-bearing material affects the inflamed sensitized cell per unit of time than affects the non-inflamed cell in the same interval. Thus the stimulus of a certain concentration of horse serum which is subliminal for a non-inflamed sensitized cell, may rise above the threshold value when an inflamed sensitized tissue is concerned, and an anaphylactic reaction occurs.

On the basis of this interplay of known conditions, the lesions observed in the sensitized and reinjected rabbits are easily explained as the expression of a primary, local, anaphylactic reaction in the skin and subcutaneous tissues of the xylol-treated ears.

Since the explanation and working hypothesis demand the interaction of antigen and sensitized cells of the skin, the phenomenon of Arthus[7] suggests itself at once as a well known example of this type of reaction, and I believe that the ear lesions described above are a variation of Arthus' phenomenon. There are, however, fundamental differences between the procedure resulting in the Arthus reaction and that causing the ear lesions described in this paper.

The Arthus reaction is a skin reaction only, and results when a repeatedly injected rabbit receives a final dose of antigen subcutaneously, the site of the last injection then developing the lesion, the severity of which depends upon the degree of sensitization. In the procedure described in this paper the sensitized animal reinjects itself where there are inflammation and edema, provided that some of the antigen is circulating, the amount circulating being so dilute that it is ineffective under ordinary conditions. Another fundamental

[7] Arthus, M., *Arch. internat. physiol.*, 1908-09, vii, 471. In this article the three notes originally published in the *Compt. rend. Soc. biol.*, 1903 and 1906 (Arthus, M., *Compt. rend. Soc. biol.*, 1903, lv, 817; Arthus, M., and Breton, M., *Compt. rend. Soc. biol.*, 1903, lv, 1478; Arthus, M., *Compt. rend. Soc. biol.*, 1906, lx, 1143), are reprinted.

difference is that the site of reaction, according to this conception, is not always the skin, as in Arthus' phenomenon, but may be any tissue capable of exhibiting an anaphylactic reaction. The reaction, therefore, may take place in the skin, the bronchial muscles, the gastrointestinal canal, the heart, the pulmonary artery, and the nerves, which are structures in which anaphylactic reactions have been described.

That the skin was chosen in the present research was a mere matter of convenience. It was selected because the changes could be directly observed at any time, thus avoiding the complication and difficulties due to indirect observation which always demands instrumental aid. As a matter of fact, preliminary series of experiments had been made on the kidney and on the intestine to test the working hypothesis, but they were abandoned because for the time being it was impossible to carry out the necessary routine work in series of animals sufficiently large to warrant the trial.

It is possible that this process of autoinoculation will aid in explaining the causation of a number of more or less temporary functional derangements of obscure origin in the human subject. In my opinion it is quite probable that sensitization to one or several alien proteins exists much more frequently in man than is suspected, the undenatured protein gaining access to the circulating juices by way of a permeable gastrointestinal mucosa or the nasal and pharyngeal mucous membranes by inhalation; the uncritical use of sera and vaccines at the present time also undoubtedly adds to the number of those sensitized unnecessarily. Severe intoxication of the sensitized individual, however, occurs but rarely since the amount of foreign protein necessary to cause obvious general symptoms on reinjection exceeds hundreds and thousands of times the amount which sensitizes. It is in such individuals that the mechanism described in this paper may come into action and produce a large variety of effects.

It is fully realized that the working hypothesis here developed at some length is not absolutely proved by the experimental data and that much more work is necessary to establish it definitely. It is believed, however, that the experimental results support the conception and that this view renders some functional abnormalities in the human subject more accessible to our understanding.

SUMMARY.

The skin irritant, xylol, when applied to the ears of sensitized and reinjected rabbits often causes a severe inflammation with the formation of crusts and destruction of tissue. Dry gangrene of the entire ear tip may result (Figs. 1, 2, and 3).

The same agent, applied in the same dosage and in the same way, to the ears of (1) normal rabbits, (2) normal rabbits injected once with horse serum (serum controls), or (3) sensitized but not reinjected rabbits, causes only a mild inflammation with more or less edema. The inflammation and edema disappear in 2 or 3 days, leaving a practically normal ear.

The ear lesions of the sensitized reinjected rabbits which develop after the application of xylol are interpreted as a primary anaphylactic reaction. This primary anaphylactic reaction is considered the result of a local autoinoculation of the ear tissues with circulating antigen. The local autoinoculation is brought about by the irritant action of the xylol which causes an inflammation and edema of the site of application. An anaphylactic reaction may now occur because the inflamed tissues are more active metabolically than normal tissues and therefore the inflamed cells are affected by more antigen per unit of time than the normal cells. A subliminal concentration of antigen for non-inflamed, sensitized cells may thus become effective when inflamed sensitized cells are concerned.

This process may theoretically occur in any tissue of a sensitized animal which can show an anaphylactic reaction, for example the intestines, lungs, heart, skin, nerves, arteries, etc. It is possible that this interplay of conditions may explain a number of functional abnormalities in the human subject.

EXPLANATION OF PLATES.

The three figures were made from a rabbit of Series 3. Sensitization, reinjection, and application of 1 cc. of commercial xylol to the left ear were carried out as described in the text.

PLATE 45.

FIG. 1. 12 days after the application of xylol; internal surface. The figure shows well the curling of the ear tip, which was dry and hard, the occurrence of hemorrhages near the gangrenous part, and the appearance of discrete blisters.

FIG. 2. 20 days after the application of xylol; dorsal surface. The dry, gangrenous tip has been removed, leaving a thickened, nodular, irregular, bluish border. Some of the crusts on the dorsal surface have also been removed to show the extensive destruction of the skin.

The dorsal surface of the right ear shows a central area where a moderate dermatitis with crust formation took place; this was due to contact of the right ear with the left shortly after application of xylol to the latter. The same effect was observed repeatedly in the sensitized reinjected groups, but not among the controls.

PLATE 46.

FIG. 3. 30 days after the application of xylol; dorsal surface. The ear has healed practically completely; note the thickened borders. The ear is largely bald but near the root some white hair is beginning to appear. This white hair gradually covered the ear stump and has remained white now for 5 months.

The right ear patch has also healed completely and shows a few white hairs. 5 months later the hair was still white.

Fig. 1.

Fig. 2.

FIG. 3.

(Auer: Local autoinoculation.)

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SYNTHESES IN THE CINCHONA SERIES. VI.

Aminoazo and Hydroxyazo Dyes Derived from Certain 5-Amino Cinchona Alkaloids and Their Quinoline Analogs.

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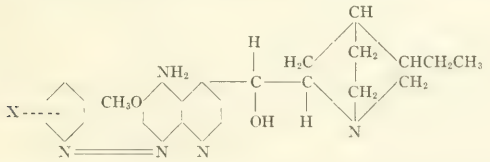
In our study of 5-amino-dihydroquinine[1] it was found to couple smoothly in dil. acetic acid solution with diazo compounds to form aminoazo dyes in which we assume the azo group to enter Position 8 in the quinoline portion of the molecule



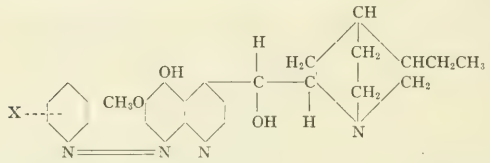
This assumption seems justified since, on the one hand, Position 8 is the only one which satisfies the usual laws of substitution for a 5-amino-6-methoxyquinoline (analogous to α-amino-β-methoxynaphthalene), and finally, since 5-aminoquinoline was also found to yield aminoazo dyes which could be reduced to 5,8-diamino-quinoline.

Of the dyes from 5-amino-dihydroquinine the phenylazo-, *p*-sulfophenylazo-, and the *p*-methoxy- and *p*-ethoxy-phenylazo-compounds were prepared and studied. 5-Amino-dihydroquinidine and 5-amino-ethyldihydrocupreine also readily yielded phenylazo compounds. These substances are usually red, well defined, crystalline compounds, forming orange-red solutions in neutral solvents and deep purple

[1] This Journal, **42**, 1485 (1920).

solutions in dilute mineral acids, except in the case of the *p*-methoxy- and ethoxy-dyes, the salts of which are a pronounced violet-blue.

If the solution of one of the amino dyes in mineral acid is boiled, a very rapid color change from a purplish-red to a brown-red is to be noted, due to the replacement of the amino group by hydroxyl, with elimination of ammonia which is readily detected on rendering alkaline. By this reaction it has been possible to prepare from the 5-amino-8-azo-cinchona dyes the corresponding 5-hydroxy compounds.



The lability of the amino group in the 5-amino compounds is surprisingly great, since boiling on the water-bath in a mixture of equal parts of 1:1 hydrochloric acid and alcohol suffices in all instances to cause complete cleavage in from 5 to 15 minutes, the alcohol being added to increase the solubility of the dye salts. Although the replacement of an amino group by hydroxyl in acid solution has been previously noted and used for preparative purposes in a number of instances, as in the preparation of α-naphthol from α-naphthylamine[2] and the dihydroxybenzenes from the phenylenediamines,[3] as a rule high temperatures have been found essential. The ease of replacement of amino by hydroxyl in our compounds more closely resembles that occurring in salts of *sym.*-triaminobenzene and its derivatives, permitting their ready and complete conversion into phloroglucinol and its derivatives at the temperature of the water-bath.[4]

It was of interest to determine whether the lability of the amino group in our compounds was due to the influence of the cinchona molecule as a whole, or whether analogous dyes obtained from 5-amino-quinoline would behave in the same way. As a matter of fact, phe-

[2] Ger. Pat. 102,358.

[3] *Ber.*, **30,** 2569 (1897).

[4] *Monatsh.*, **18,** 755 (1897); **19,** 223, 236, 249 (1898); **21,** 39 (1900).

nylazo-5-aminoquinoline yielded phenylazo-5-hydroxyquinoline, but it required a definitely longer period of heating to bring about complete cleavage. On the other hand, the dyes from 5-amino-6-methoxyquinoline, which afforded a closer parallel to the 5-amino-dihydroquinine dyes, were found to be as easily converted into the hydroxy dyes as the alkaloidal derivatives themselves. It would seem, therefore, that the methoxy or ethoxy group in Position 6 contributes markedly to the lability of the amino groups in these compounds.

The hydroxyazo dyes are also substances with excellent properties and crystallize very readily. In spite of the hydroxyl group they do not dissolve in dil. alkali, but after making an alcoholic solution alkaline it may be diluted freely without precipitating the dye, subsequent addition of ammonium chloride being, however, sufficient to cause precipitation.

On reduction the new cinchona and quinoline dyes yield amino compounds in which interesting relationships have been observed in the replacement of the amino group by hydroxyl. These studies are still in progress, and we shall describe them in a subsequent communication.

I.

5-Amino-8-phenylazo-dihydroquinine.—One g. of aniline dissolved in 30 cc. of normal hydrochloric acid was diazotized with 10 cc. of normal sodium nitrite, with chilling. A solution of 3.4 g. of 5-amino-dihydroquinine[5] in 20 cc. of normal acetic acid was diluted to 200 cc., treated with 20 cc. of saturated sodium acetate solution, chilled to about 10°, and slowly treated, with turbining, with the diazo solution. The original orange-red solution rapidly changed to a deep red, and after 15 minutes the dye was precipitated as deep red flocks by the addition of an excess of ammonia, filtered off, and washed with water. On taking up the moist dye in hot alcohol it dissolved partially but almost immediately crystallized. Recrystallized from 95% alcohol, the dye gradually separated as glistening, red needles containing approximately one molecule of water of crystallization when air-dried. The yield was 2.2 g. The air-dry substance, melts at 155–7° with preliminary darkening and sintering. It dissolves

[5] *Loc. cit.*

sparingly in the cold in methyl and ethyl alcohols, benzene, and chloroform, but readily on warming, forming red solutions. It is sparingly soluble in acetone and ether. Solutions in dilute acids are reddish-purple, and in conc. sulfuric acid an intense purple. After dissolving the dye in 10% hydrochloric acid the hydrochloride deposits on rubbing as deep brown, microscopic needles.

Subs. (air-dry), 0.4760: loss, 0.0227 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{26}H_{31}O_2N_5.H_2O$: H_2O, 3.89. Found: 4.77.
Subs., anhydrous, 0.1109: 15.0 cc. N (24.0°, 772 mm.).
Calc. for $C_{26}H_{31}O_2N_5$: N, 15.72. Found: 15.79.

5-Hydroxy-8-phenylazo-dihydroquinine.—5 g. of the preceding amino-azo dye were treated with a hot mixture of 25 cc. of alcohol and 25 cc. of 1:1 hydrochloric acid and the resulting deep purple solution heated on the water-bath. The mixture rapidly changed to a dark brown and set to a jelly which slowly changed to long, brown needles of the hydrochloride of the hydroxyazo dye. After 15 minutes the mass was diluted with water, chilled, and treated with an excess of ammonia, yielding a purple mass of the free base. After filtering and washing with water, the dye was taken up in hot alcohol, quickly separating as brownish yellow, microscopic leaflets in a yield of 4.2 g. On dissolving in hot water with the aid of dil. sulfuric acid, filtering, adding an equal volume of alcohol, and then treating hot with a slight excess of ammonia, the dye quickly crystallized as lustrous, orange-brown platelets which, when rapidly heated to 140° and then slowly, gradually sinter together and effervesce at 145–8°. When air-dry it contains one molecule of water of crystallization which requires heating at 100° *in vacuo* for its complete removal. The dye is readily soluble in hot benzene and chloroform, less readily in hot methyl and ethyl alcohols, and very sparingly in hot acetone, forming orange-red solutions, and is almost insoluble in ether. It dissolves in dil. sulfuric acid with an orange-red color, and in the conc. acid with a cherry-red shade, appearing purple in thin layers. In dil. hydrochloric acid it forms an insoluble, gelatinous salt.

Subs. (air-dry), 0.2940: loss, 0.0122 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{26}H_{30}O_3N_4.H_2O$: H_2O, 3.88. Found: 4.15.
Subs., anhydrous, 0.1296: 14.2 cc. N (25.5°, 754 mm.).
Calc. for $C_{26}H_{30}O_3N_4$: N, 12.55. Found: 12.43.

5-Amino-8-(p-sulfophenylazo)-dihydroquinine.—A solution of diazotized sulfanilic acid was rapidly added to 3.4 g. of amino-dihydroquinine under the same conditions as previously given. A dark red-brown, amorphous precipitate of the dye formed at once, and was filtered off and washed with water. On treating with hot alcohol it dissolved partially, but almost immediately changed to orange, microscopic needles. The yield was 4 g. after washing with acetone. On recrystallizing from 50% alcohol it formed delicate, minute, orange needles containing about 0.5 molecule of water of crystallization. In working with large quantities of the dye it was found more convenient to dissolve it in the minimum amount of dil. alkali, add an equal volume of alcohol, warm, and reacidify with an equivalent amount of acetic acid. The air-dry substance darkens above 180°, decomposes at about 245°, and is practically insoluble in the usual neutral solvents. In dil. mineral acids it gives a deep cherry-red solution, appearing purple in thin layers, and when not too dilute sulfuric or hydrochloric acid is used the difficultly soluble salt separates as a dark-colored, crystalline powder. In conc. sulfuric acid the solution is bright red, appearing purple in thin layers. The solution in 50% acetic acid is quickly reduced by stannous chloride to diamino-dihydroquinine, which will be described in a later communication.

Subs. (air-dry), 0.5041: loss, 0.0114 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{26}H_{31}O_5N_5S.0.5H_2O$: H_2O, 1.68. Found: 2.26.
Subs. (anhydrous), 0.1188: 13.4 cc. N (23.5°, 772 mm.).
Calc. for $C_{26}H_{31}O_5N_5S$: N, 13.32. Found: 13.18.

5-Hydroxy-8-(p-sulfophenylazo)-dihydroquinine.—Two g. of the amino-azo dye were treated with a hot mixture of 20 cc. of alcohol and 20 cc. of 10% hydrochloric acid and boiled on the water-bath. The solution almost immediately set to a jelly which soon began to change to the crystalline salt of the new dye. After 5–10 minutes the mixture was made alkaline with dil. sodium hydroxide, a strong odor of ammonia being at once noticeable. The deep purple solution was treated with an excess of acetic acid, yielding 1.8 g. of glistening, salmon-colored needles on diluting and rubbing. Recrystallized from 50% alcohol it separated as delicate, lustrous, scarlet needles containing approximately one molecule of water of crystallization. The

dye decomposes at 275° with preliminary darkening, and is fairly readily soluble in hot 50% alcohol and methyl alcohol, sparingly in hot water and hot alcohol, and practically insoluble in the usual neutral solvents. With dil. hydrochloric and sulfuric acid it forms brown-orange salts and dissolves in conc. sulfuric acid with a deep scarlet color, appearing violet in thin layers and changing to orange on dilution with water. In alkali the color is purplish red.

Subs. (air-dry), 0.4260: loss, 0.0164 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{26}H_{30}O_6N_4S.H_2O$: H_2O, 3.31. Found: 3.85.
Subs. (anhydrous), 0.1358: 12.8 cc. N (24.5°, 750 mm.).
Calc. for $C_{26}H_{30}O_6N_4S$: N, 10.63. Found: 10.67.

5-Amino-8-(p-methoxyphenylazo)-dihydroquinine.—Although diazotized *p*-anisidine failed to yield a dye in dil. acetic acid as in previous cases, the following conditions were successfully employed. 1.23 g. of *p*-anisidine in 15 cc. of 2 *N* hydrochloric acid were diazotized at 0° with 2 cc. of 5 *N* sodium nitrite. 3.4 g. of amino-dihydroquinine in 10 cc. of *N* acetic acid were then added and the mixture immediately treated with sufficient saturated sodium acetate solution to bind the free hydrochloric acid. After 2 hours the mixture, from which an almost black tar had separated, was diluted with enough alcohol to form a homogeneous solution, warmed, and made alkaline with ammonia. The dye gradually separated on rubbing, and after filtering and washing with cold alcohol the yield was 2.8 g. Recrystallized twice from 95% alcohol it forms red, microscopic needles and platelets which melt slowly at 150–3°. It dissolves readily with an orange-red color in cold chloroform, on boiling in methyl or ethyl alcohol, acetone, or benzene, and but sparingly in ether. In strong mineral acid it gives a deep violet-blue color which changes to brownish-red on dilution, a phenomenon apparently due to hydrolysis of the polyacid salts originally formed. In conc. sulfuric acid the color is deep red, appearing violet in thin layers. As in the case of the other amino cinchona dyes, the deep violet-blue color in acid changes to a purplish-red on boiling, with simultaneous cleavage of ammonia.

Subs., 0.1440: 18.6 cc. N (25.0°, 765 mm.).
Calc. for $C_{27}H_{33}O_3N_5$: N, 14.72. Found: 14.90.

5-Amino-8-(p-ethoxyphenylazo)-dihydroquinine.—Under the conditions used with *p*-anisidine 1.75 g. of *p*-phenetidine hydrochloride also yielded a dark brown tar. After decanting the supernatant liquid the dye was dissolved in alcohol and treated with ammonia. On rubbing, the base crystallized and was filtered off and washed with 85% alcohol. The yield was 2.6 g. Recrystallized first from a relatively large volume of 95% alcohol, then by dissolving in warm chloroform and adding a little ligroin, it was gradually deposited as long, thin, narrow, orange platelets, often grouped in rosets. It apparently contains solvent of crystallization which is slowly lost on exposure to the air or on drying *in vacuo* at 100°. The dye decomposes at 202–3° with preliminary darkening, and dissolves readily in warm chloroform, much less easily in boiling methyl and ethyl alcohols, and quite sparingly in hot benzene. It is very difficultly soluble in acetone and ether and insoluble in water and ligroin. The solution in dil. mineral acids is blue-violet, changing on dilution to a dark wine-red, owing to hydrolysis of the polyacid salts. Conc. sulfuric acid gives a deep purple color. In boiling dilute acids it also yields an hydroxyazo dye, which was not studied. For analysis the substance was heated *in vacuo* at 80° over sulfuric acid.

Subs., 0.1224: 15.8 cc. N (22.0°, 746 mm.).
Calc. for $C_{28}H_{35}O_3N_5$: N, 14.30. Found: 14.67.

5-Amino-8-phenylazo-dihydroquinidine.—This substance was prepared exactly as was the isomeric dihydroquinine dye. The amorphous product, taken up in hot, dry acetone, crystallized on cooling and rubbing, the mother liquor yielding additional amounts on dilution with water to incipient turbidity. The total yield was 2.7 g. Recrystallized from 85% alcohol the base separates as rosets of orange-red leaflets when deposited rapidly, or as a dark red crust when allowed to crystallize slowly. The air-dry compound contains 1.5 molecules of water of crystallization and melts and intumesces at 135° with preliminary sintering. It turns dark brown on dehydrating, and then melts to a tar at about 140–5°, intumescing at about 155°. It is readily soluble in absolute alcohol, chloroform, warm benzene, and warm acetone, forming deep red solutions. The dye dissolves in 10% hydrochloric acid with a deep purplish-red color, the hydrochloride

separating on rubbing as brown, hair-like needles with a greenish luster. The solution in conc. sulfuric acid is an intense violet.

Subs. (air-dry), 0.4887: loss, 0.0276 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{26}H_{31}O_2N_5.1.5H_2O$: H_2O, 5.72. Found: 5.65.
Subs. (anhydrous), 0.1232: 16.6 cc. N (23.5°, 771 mm.).
Calc. for $C_{26}H_{31}O_2N_5$: N, 15.73. Found: 15.74.

5-Amino-8-phenylazo-ethyldihydrocupreine.—Treated as in the case of amino-dihydroquinine, 3.55 g. of amino-optochin[6] yielded 3 g. of the aminoazo dye. The base separates from 95% alcohol as red, glistening needles containing one molecule of water of crystallization. It starts to sinter at 145° and shrinks together and melts at 150–5°. It is sparingly soluble in cold methyl and ethyl alcohols, benzene, and chloroform, but readily on warming, forming deep reddish-orange solutions. It is difficultly soluble in acetone and very sparingly in ether. The solutions in dilute acids are deep reddish-purple in color and purple in conc. sulfuric acid.

Subs. (air-dry), 0.4098: loss, 0.0155 *in vacuo* at 100° over H_2SO_4.
Calc. for $C_{27}H_{33}O_2N_5.H_2O$: H_2O, 3.77. Found: 3.78.
Subs. (anhydrous), 0.1333: 17.25 cc. N (21.5°, 768 mm.).
Calc. for $C_{27}H_{33}O_2N_5$: N, 15.23. Found: 15.17.

5-Hydroxy-8-phenylazo-ethyldihydrocupreine.—The deep purple solution in hot aqueous-alcoholic hydrochloric acid rapidly changed to deep red. On dilution with alcohol, adding ammonia, and finally water until turbid, the crystalline base was readily obtained. Recrystallized twice from 85% alcohol the dye forms lustrous, red needles containing 3.5 molecules of water of crystallization. It starts to sinter at 85°, shrinks together and melts at 90.5°, and is soluble in alcohol, acetone, ether, and hot benzene, forming red solutions. It dissolves in 10% sulfuric acid with a deep brown-red color, and in the conc. acid with a deep purple color. The anhydrous dye softens above 100°, sinters to a tar at 110–20°, and melts completely, with decomposition, at about 200°.

Subs. (air-dry), 0.6035: loss, 0.0715 *in vacuo* at 80° over H_2SO_4.
Calc. for $C_{27}H_{32}O_3N_4.3.5H_2O$: H_2O, 12.03. Found: 11.84.
Subs. (anhydrous), 0.1181: 12.6 cc. N (26.5°, 761 mm.).
Calc. for $C_{27}H_{32}O_3N_4$: N, 12.17. Found: 12.18.

[6] THIS JOURNAL, **42**, 1486 (1920).

II.

5-Amino-8-phenylazoquinoline.—5-Aminoquinoline was coupled with diazotized aniline and the base isolated as in the case of the cinchona derivatives. The dye separates from 50% alcohol as arborescent masses of garnet-colored leaflets which melt at 209–11°. It is soluble in alcohol, acetone, chloroform, hot benzene, and less readily in ether. Its solution in dilute acid is deep red, appearing purple in thin layers and changing to orange in dilution. In conc. sulfuric acid the color is deep red.

Subs., 0.1224: 23.8 cc. N (22.5°, 767 mm.).
Calc. for $C_{15}H_{12}N_4$: N, 22.57. Found: 22.68.

On reduction with ammonium sulfide the analogous *p*-sulfophenyl-5-aminoquinoline, which was similarly prepared, yields 5,8-diaminoquinoline, melting at 161–3° and corresponding in its properties with those described by Claus and Kramer,[7] who give the uncorrected melting point of 156°.

5-Hydroxy-8-phenylazoquinoline.—The aminoazo dye was boiled with 100 parts of 10% hydrochloric acid, a portion of the amino hydrochloride separating at once. The color of the deep purplish-red solution slowly changed, with deposition of the brown hydrochloride of the hydroxyazo dye. About one hour was required for the complete disappearance of the purple color. The collected salt was suspended in 50% alcohol and the base liberated with ammonia. After several recrystallizations from alcohol the dye forms slightly purplish-red, lustrous needles, which melt at 164–5° with preliminary sintering. It is easily soluble in chloroform, fairly readily in hot alcohol, benzene and acetone, and but sparingly in ether. It gives a scarlet solution in conc. sulfuric acid, appearing purple in thin layers and changing to orange on dilution.

Subs., 0.1191: 17.6 cc. N (25.0°, 758 mm.).
Calc. for $C_{15}H_{11}ON_3$: N, 16.86. Found: 16.88.

5-Amino-6-methoxyquinoline.—The 5-nitro-6-methoxyquinoline[8] used for this preparation was easily obtained in excellent yield by nitrating 6-methoxyquinoline with fuming nitric acid at 0°.

[7] Claus and Kramer, *Ber.*, **18,** 1247 (1885).
[8] Decker and Engler, *Ber.*, **42,** 1739 (1909).

28 g. of the nitro compound were suspended in 200 cc. of 1:1 hydrochloric acid, treated with 125 g. of stannous chloride, and heated on the water-bath for one hour. A clear, deep red solution was soon obtained, and the free base was finally precipitated with excess alkali as a yellow oil which crystallized on cooling. Extraction of the mother liquor with ether yielded an additional quantity, the total amounting to 90% of that theoretically possible. Recrystallized first from benzene and then from ligroin, it forms yellow, arborescent platelets and needles which melt at 154–6° (corr.) with slight preliminary sintering. It has a faint odor, resembling that of anise, and dissolves readily in alcohol, acetone, chloroform, and hot benzene, but less easily in ether. Its solution in dilute acid is reddish-orange.

Subs., 0.1393: 19.2 cc. N (22.5°, 765 mm.).
Calc. for $C_{10}H_{10}ON_2$: N, 16.08. Found: 16.02.

5-Amino-6-methoxy-8-phenylazoquinoline.—Obtained in the usual way, the crude dye was precipitated by ammonia as red, amorphous flocks which soon crystallized on dissolving in a small volume of acetone. It separates slowly and incompletely from 85% alcohol as thin, deep red rods which exhibit a green reflex when dry, and melt at 163–4°. It dissolves, especially on warming, in the usual solvents, forming deep orange-red solutions. In dilute acids the color is purplish-red, and in conc. sulfuric acid a deep purple.

Subs., 0.1062: 18.6 cc. N (25.0°, 758 mm.).
Calc. for $C_{16}H_{14}ON_4$: N, 20.12. Found: 20.02.

5-Hydroxy-6-methoxy-8-phenylazoquinoline.—On treating the amino-azo dye with 30 parts of a mixture of equal amounts of alcohol and 1:1 hydrochloric acid a deep purple mass of the salt separated and gradually dissolved when heated on the water-bath, the color of the solution changing to red, with subsequent deposition of the brown salt of the hydroxy dye. This was converted into the base as usual, and this recrystallized from alcohol, forming broad, scarlet, often curved needles which melt at 181–3°. It is readily soluble in chloroform and in the other usual solvents on warming. It is but sparingly soluble in cold dilute mineral acids, and on boiling forms deep red-

orange solutions. The color in conc. sulfuric acid is wine-red, appearing purple in thin layers.

Subs., 0.1133: 15.0 cc. N (24.5°, 759 mm.).
Calc. for $C_{16}H_{13}O_2N_3$: N, 15.04. Found: 15.16.

SUMMARY.

It is shown that 5-amino-dihydroquinine couples readily with diazotized aromatic amines to form crystalline azo dyes in which the amino group is remarkably labile boiling for a few minutes with dil. mineral acid being sufficient to replace the amino group by the hydroxyl group, with elimination of ammonia. The resulting hydroxy-azo dyes are also easily crystallizable substances. Similar results were obtained using 5-amino-dihydroquinidine and 5-amino-ethyldihydrocupreine (amino-optochin) as couplers. Since it was of interest to determine whether the observed phenomena were a function of the quinoline portion of the cinchona structure, parallel experiments were run with 5-aminoquinoline and 5-amino-6-methoxyquinoline. Both of these bases coupled as did the amino alkaloids, and the amino group of the resulting dyes was also readily eliminated and replaced by hydroxyl, the lability being greater in the case of the methoxy derivatives.

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THE SIGNIFICANCE OF THE HYDROGEN ION CONCENTRATION FOR THE DIGESTION OF PROTEINS BY PEPSIN.

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One of the most striking peculiarities of enzyme action is the fact that the activity of the enzyme is limited to a definite range of acidity. If the solution is more or less acid than this the enzyme is practically inactive. Sörensen[1] showed that for a number of enzymes the determining factor was the hydrogen ion concentration and not the total acidity of the solution.

In attempting to account for this phenomenon it has usually been assumed that the influence of the hydrogen ion concentration was exerted upon the enzyme. Michaelis[2] pointed out, in the case of many enzymes, that if the activity of the enzyme was plotted against the hydrogen ion concentration of the solution the curve resembled strikingly that obtained when the ionization of a salt of a weak base and a strong acid was plotted in the same way. He concluded therefore that enzymes were weak bases or acids which formed salts with the acids or bases of the solution. These salts then dissociated into ions and the ions so formed were the active agents in the reaction. A similar explanation had already been proposed independently by Loeb[3] and by Nasse.[4] Michaelis' work rendered the hypothesis quite plausible. In the case of pepsin, however, it meets with several serious objections. In the first place, one of the strongest points of Michaelis' experiments was the fact that pepsin was found to have an isoelectric point at about pH 3.0 which agreed fairly well with the

[1] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

[2] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914, 58.

[3] Loeb, J., *Biochem. Z., 1909*, xix, 534.

[4] Nasse, O., *Malys Jahrb., 1894* xxiv, 718.

theory. Pekelharing and Ringer,[5] however, showed that in solutions of pure pepsin (prepared by Pekelharing's method from gastric juice) the pepsin was always negatively charged. This objection may of course be met by the statement that the pepsin under the actual conditions of hydrolysis (*i.e.* when in the protein solution) is not pure but is combined with some other substance and it is the ionization of this compound which determines the activity of the enzyme. An explanation similar to this has been offered by Michaelis.[6] The author[7] has shown, however, that pepsin combined with peptone or other decomposition products of the proteins is inactive and that it is only the free pepsin which takes part in the reaction. It was also found[8] that no positively charged pepsin could be found on the alkaline side of pH 3.3. Pepsin retains its activity up to pH 5, however, so that it seems unlikely that only positively charged pepsin is active, as assumed by Michaelis.

A second objection to Michaelis' view is the fact that the optimum hydrogen ion concentration for the activity of pepsin is found to vary with the substrate. This point has been emphasized by Long and Hull[9] (for trypsin) and by Ringer.[10] From Michaelis' point of view it is difficult to see how this can be. Neither of these objections, however, can in the author's opinion be considered as conclusive evidence against Michaelis' hypothesis. It could be stated for instance that pepsin contained several enzymes, one for each substrate and each with a different optimum. It seems simpler, however, to assume that the hydrogen ion concentration affects the condition of the substrate rather than the enzyme. This hypothesis has the advantage that it also accounts for the peculiar relation between the concen-

[5] Pekelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

[6] Michaelis, L., *Deutsch. med. Woch.*, 1920, xlvi, 1.

[7] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 471.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 468.

[9] Long, J. H., and Hull, M., *J. Am, Chem. Soc.*, 1917, xxxix, 1051. The same statement is made by Hedin and Hammerstein. The author has been unable to find the original work on which this statement is based. *Cf.* Hammerstein, O., and Hedin, S. G., A text-book of physiological chemistry, translated by Mandel, J. A., New York, 8th edition, 1915, 471. See also Abderhalden, E., and Fodor. A., *Fermentforsch.*, 1914–16, i, 591.

[10] Ringer, W. E., *Kolloid. Z.*, 1916, xix, 253.

tration of the substrate and the rate of hydrolysis. Experiments described in a former paper[11] show that the rate of hydrolysis of protein solutions of varying concentration but the same pH was directly proportional to the amount of ionized protein present in the solution but not to the total concentration of protein. They agree therefore with the hypothesis that the ionized protein is the form which takes part in the reaction. If this explanation is correct it follows that the optimum hydrogen ion concentration for the activity of pepsin is also due to the increased ionization of the protein and must coincide with the hydrogen ion concentration at which the protein solution contains the greatest number of protein ions. (It was first suggested by Pauli[12] that the ionized protein was the form which was attacked. Euler[13] and Arrhenius[14] have made a similar suggestion. Ringer[15] considers also that the ionization of the substrate has an influence on the rate of digestion at least in the later stages.) It should be possible therefore to determine the optimum degree of acidity for pepsin digestion by measuring the conductivity of the protein solution. It will be shown below that this is true. It will further be shown that the range of hydrogen ion concentration in which the enzyme is active shifts in the same sense as the conductivity of the protein solution when a protein of different isoelectric point is used, and also that when the protein is insoluble the enzyme combines with it only over that range of hydrogen ion concentration in which the enzyme is active and in which the protein is ionized.

The Influence of the Isoelectric Point of the Protein on the Activity of Pepsin at Different Hydrogen Ion Concentrations.

Ringer[10] has already shown that the optimum hydrogen ion concentration for the digestion of proteins by pepsin varies somewhat with the protein hydrolyzed and with the acid used. He accounts for this phenomenon by the assumption that the hydration of the

[11] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 595.
[12] Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483.
[13] Euler, H., Allgemeine Chemie der Enzymes, Wiesbaden, 1910.
[14] Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.
[15] Ringer, W. E., *Arch. Neerl. Physiol.*, 1917–18, ii, 571.

protein determines the ease with which it is attacked by the enzyme. The viscosity is assumed to be a measure of the hydration. The same explanation has been proposed by Brücke,[16] by Pfliederer,[17] and recently by Traube.[18] The writer has been able to show,[19] however, that gelatin digests at the same rate in sulfuric or hydrochloric acid solution (provided the pH is the same) although the swelling, which Ringer considers a measure of the hydration, is much greater in hydrochloric than in sulfuric acid. It was also found[11] that the rate of digestion of egg albumin solutions decreased as the viscosity increased with the age of the solution instead of increasing as would be expected if the rate of digestion was determined by the hydration of the protein (as shown by the viscosity). Loeb[20] has shown that the ionization of the protein and the viscosity and swelling are all approximately proportional for a small range of acidity to the acid side of the isoelectric point. The maximum for the swelling and viscosity, however, occurs at about pH 3.4 whereas that for the ionization is much further to the acid side and agrees very well for that of the rate of digestion. This question will be discussed more fully below. It is clear, however, that in certain cases the swelling or viscosity and the ionization and rate of digestion may all be proportional. It would seem from the experiments described here that the determining factor for the rate of digestion is the ionization of the protein, and the swelling and viscosity are secondary characteristics which are probably also connected with the ionization.

It is known that, with most proteins, pepsin becomes inactive at a pH of about 4.5. This cannot be ascribed to the destruction of the enzyme since the author[8] found pepsin to be more stable in this range of acidity than at any other. The ionization of most proteins is very slight at this pH, however, so that it would be expected (from the hypothesis that it is the protein ion which is attacked by the enzyme) that little or no hydrolysis should occur at this point. Oxy-

[16] Brücke, E., *Sitzungsb. k. Akad. Wissensch. Math-naturw. Cl., Wien.*, 1859, xxxvii, 131.

[17] Pfliederer, R., *Arch. ges. Physiol.*, 1897, lxvi, 605.

[18] Traube, M., *Deutsch. med. Woch.*, 1919, xxvii,

[19] Northrop, J. H., *J. Gen. Physiol.*, 1918–19, i, 607.

[20] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39; 1920–21, iii, 85.

hemoglobin, however, is isoelectric at a pH of about 6.8 (Michaelis[2]) so that it must be quite largely present as a salt and therefore ionized at a pH of 4.5. It would be predicted then, according to the hypothesis that the amount of protein ions present determines the rate of digestion of the protein, that hemoglobin should be digested by pepsin at pH 4.5 more rapidly than is egg albumin or gelatin at the same pH.

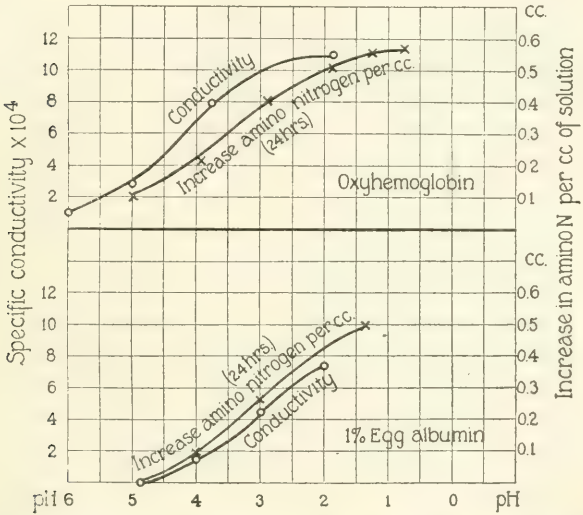

FIG. 1. Influence of pH on conductivity and rate of digestion of egg albumin and oxyhemoglobin solutions.

In order to test this prediction, parallel experiments were made to determine the rate of digestion and the conductivity of hemoglobin and egg albumin solutions at various hydrogen ion concentrations. The results of such an experiment are shown graphically in Fig. 1. It is clear that the conductivity and digestion curves, for each protein, as plotted against the pH of the solution are approximately parallel

and also that the curves for the digestion and conductivity of the hemoglobin fall further to the left (*i.e.* to the alkaline side) than do the curves for the egg albumin.

The experiments cannot be considered as showing quantitative agreement between the rate of digestion and the conductivity of the solution since the digestion curve is given as the amount of protein decomposed in a certain time—a quantity which is not connected in any simple way with the rate of digestion. They are further complicated by the fact that the digestion in the region of the optimum acidity represents approximately 50 per cent of the complete digestion of the protein and therefore probably includes the secondary splitting of some of the primary products of the hydrolysis, and not purely the action on the protein itself. The conductivity on the other hand was measured on the protein solution itself. It is not possible to carry the digestion curve much beyond pH 5.0 owing to the rapid destruction of the enzyme.

EXPERIMENTAL.

Egg Albumin.—The egg albumin was crystallized three times as described by Hopkins and Pinkus[21] and then dialyzed under pressure of about 150 cm. of water at pH 4.8 until the specific conductivity of the solution was less than 1×10^{-4} reciprocal ohms. The solution was then diluted to 2 per cent with water. Increasing amounts of HCl were added to a series of 50 cc. portions of this solution and the total volume made up to 100 cc. 1 cc. of 2 per cent pepsin was then added to 25 cc. of these solutions and placed at 25°C. 1 cc. of the solution was analyzed by the Van Slyke[22] method for amino nitrogen after 0, 8, 24, and 36 hours. The curve given is the increase in cubic centimeters of amino nitrogen per cubic centimeter of solution after 24 hours. The 8 and 36 hour curves were similar.

Conductivity.—1 cc. of inactivated pepsin was added to another 25 cc. portion of the above solutions and the conductivity and pH of the solution were measured at 25°C. The conductivity of the egg albumin salt was determined from the conductivity of the solution by subtracting from the observed conductivity the conductivity of HCl of the same pH (Northrop[11]).

Oxyhemoglobin.—Erythrocytes from fresh defibrinated ox blood were washed with 7.8 per cent glucose solution until the conductivity of the suspension was less than 1×10^{-4} reciprocal ohms. The cells were then laked with ether, separated from the excess ether, and the ether in the solution removed *in vacuo*. The solution was then diluted to contain about 1 cc. of amino nitrogen per cubic centimeter as determined by the Van Slyke method. The conductivity of this

[21] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

[22] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

solution was about 1×10^{-5} reciprocal ohms. Increasing amounts of HCl were added to 50 cc. portions of this solution and the total volume made up to 100 cc. The conductivity and digestion of the solution were then determined as described for the egg albumin.

The Optimum Hydrogen Ion Concentration for Pepsin Digestion.

The optimum hydrogen ion concentration for the activity of pepsin has been determined many times. All the methods used for following the digestion, however, have depended on the change in some physical property of the protein. It seemed of interest therefore to determine the optimum degree of acidity for the reaction when the hydrolysis was followed by means of the increase in amino nitrogen, which

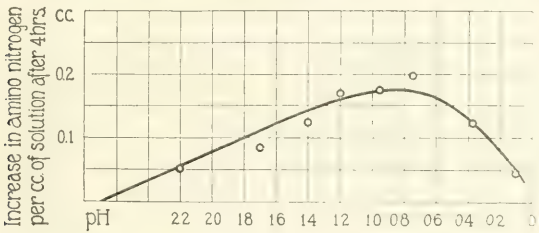

FIG. 2. Influence of pH on the rate of digestion of egg albumin.

probably represents correctly the actual course of the digestion. The method has the disadvantage, however, that only comparatively large changes can be followed. The results of an experiment made with egg albumin solutions of different pH (adjusted with HCl) are given in Fig. 2.

The time of digestion was 4 hours. The figure shows that the optimum acidity for the digestion as determined by the increase in amino nitrogen is at about pH 1.0 (0.1 N). This is slightly more acid than that found by Sörensen,[1] Michaelis and Mendelssohn,[23] or Okada,[24] and much more acid than that found by Ringer.[15] It must be remembered,

[23] Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 1.
[24] Okada, S., *Biochem. J.*, 1916, x, 126.

however, that the chemical changes followed by the increase in amino nitrogen represent much more complete hydrolysis than those followed by the other authors. The curve therefore probably does not represent the correct optimum for the digestion of the protein itself but probably also the digestion of some of the primary products. The careful work of Ringer has shown that the optimum zone for the digestion of these products extends further to the acid side than the zone for the digestion of the protein itself. This probably accounts for the difference in the optimum found by the different methods and agrees with the results of Sörensen[1] who found that the optimum shifts to the acid side with more complete digestion.

The Effect of Adding Salt with a Common Ion to a Solution Already Containing the Optimum Amount of Acid.

It will be noted from the curve (Fig. 2) that the amount of digestion increases with increasing amounts of acid in the solution until the hydrogen ion concentration is about 0.1 N and then decreases. According to the hypothesis that it is the ionized protein which is hydrolyzed by the pepsin, the increase in digestion from pH 4.0 to 1.0 is due to the fact that as acid is added to the albumin more protein salt and hence more protein ions are formed in the solution, until all the albumin is present as salt. The addition of a further amount of acid serves to depress the concentration of protein ions again due to the effect of the common ion. According to this mechanism the hydrogen ion concentration is the determining factor on the alkaline side of the optimum while on the acid side the concentration of the anion is the determining factor. It can be predicted therefore that if a solution of a salt (having the same anion as the acid) is added to a solution of the protein which already contains the optimum amount of acid, the depressing effect of the salt on the digestion should be the same as if excess acid had been added, provided the final anion concentration is the same. The conductivity of the albumin salt should also be diminished. In the case of egg albumin this cannot be experimentally verified owing to the fact that the albumin precipitates under these conditions, and also since the conductivity of the protein in such strongly acid solutions is so small, compared to

the total conductivity, as to render the measurement very uncertain. It will be shown later, however, that in the case of gelatin the decrease in conductivity can be followed and is proportional to the decrease in the rate of digestion.

TABLE I.

Increase in Amino Nitrogen per Cc. of Solution Containing Normal Total Chlorine Concentration Furnished by Different Salts.

Original solution 0.5 N HCl.
Made up to 1.0 N chloride concentration with salt noted.

Salt.	pH	Increase in NH_2 nitrogen per cc after 6 hrs. at 25°C.
		cc.
O	0.42	0.25 0.26
NaCl	0.40	0.15 0.13
KCl	0.42	0.15 0.15
$CaCl_2$	0.41	0.17 0.15
$MgCl_2$	0.42	0.12 0.11
$SrCl_2$	0.42	0.17 0.18
$AlCl_3$	0.40	0.10 0.11
HCl	0.13	0.13 0.14

Table I contains a summary of the results of such an experiment in which a series of egg albumin solutions all containing a total chlorine ion concentration of 0.5 N and at a pH of 0.42 were brought to a total chlorine ion concentration of 1.0 N by the addition of the salts noted or excess acid. The final solutions therefore were all

1.0 N in respect to the chlorine ion but those which had been brought to this concentration by the addition of salts were of course much less acid than the one to which excess acid had been added. The amount of digestion in all the solutions containing the same chlorine ion concentration was approximately the same, however. This result indicates that the controlling factor on the acid side of the optimum is the anion concentration and not the hydrogen ion concentration. As a corollary of this it can be stated that the addition of salt to a protein solution will cause the optimum hydrogen ion concentration for digestion to be shifted to the alkaline side. This was the effect noted by Michaelis and Mendelssohn.[23]

The above question has recently been examined by Gyemant.[25] This author found, however, the optimum pH for digestion remained at about pH 2.0 even though the anion concentration was the same in all the solutions. He concludes therefore that the decrease in the rate of digestion on the acid side of the optimum is due to the influence of the hydrogen ion on the pepsin as proposed by Michaelis.

The experiments described in this paper are complicated by the fact that the egg albumin was partially precipitated by the high concentrations of salt and acid used. This may account for the difference between the present results and those of Gyemant. The discrepancy may also be due to the fact that Gyemant followed the reaction by means of the increase in non-protein nitrogen whereas the author used the increase in amino nitrogen. In view of Gyemant's results and of the complicating factor of precipitation in the present experiments, they cannot be considered as conclusive evidence in favor of the view that the anion alone affects the digestion on the acid side of the optimum. It is possible that both ions are active. It appears to the author, however, that the action is exerted on the protein rather than the enzyme in view of the fact that different proteins show slightly different optimum pH, and of the close connection between the conductivity and rate of digestion of gelatin solutions (as described below in this paper).

The Conductivity and Rate of Digestion of Gelatin Solutions.

It was mentioned above that determinations of the conductivity of egg albumin solutions in strongly acid solution were made uncertain owing to the precipitation of the protein. This difficulty is not encountered with gelatin. Gelatin possesses the further advantage that the rate of digestion in the very early stages may be easily fol-

[25] Gyemant, A., *Biochem. Z.*, 1920, cv, 155.

lowed by noting the time necessary to cause a certain degree of liquefaction of the gelatin.

A series of gelatin solutions, containing 5 per cent dry weight of gelatin and adjusted to various hydrogen ion concentrations by means of HCl, were prepared. The gelatin had previously been purified as described by Loeb.[26] The conductivity of the solutions and the time necessary for them to reach an easily determined degree of liquefaction were then determined. The reciprocal of this time is plotted in the curve as the rate. Fig. 3 and Table II show the result of a typical experiment of this kind. It is clear that the rate of diges-

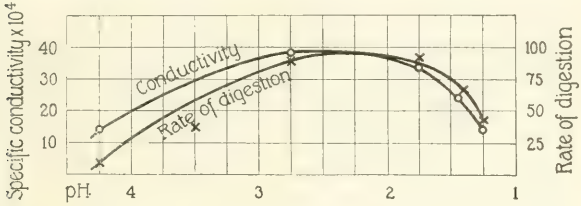

FIG. 3. Influence of pH on the rate of digestion and conductivity of gelatin solutions.

tion and the conductivity of the solution have their maximum value at the same hydrogen ion concentration, and that the curves are nearly parallel throughout. The rate of digestion decreases slightly more rapidly than the conductivity of the solution on the alkaline side of the optimum and slightly less rapidly on the acid side. This peculiarity was noted in all the experiments made and can hardly be ascribed to experimental errors. It shows that the digestion on the alkaline side of the optimum is slightly less rapid than would be predicted from the conductivity data and that it is slightly more rapid on the acid side. The divergence on the acid side is due to the fact that in such strongly acid solutions the acid itself has some action on the protein as was shown by control experiments without any pepsin. The correction is too uncertain to be applied to the figures, however.

[26] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

It can only be said that such a correction is necessary and that it would be in the right sense. The divergence on the alkaline side is probably due to the fact that the amount of hydrolysis selected as the end-point represented too great a percentage change in the original substrate concentration to assume that the substrate concentration remained constant during the course of the experiment.

TABLE II.

pH, Conductivity, and Rate of Digestion of Gelatin Solutions.

Gelatin, 5 per cent dry weight in solution of total (approximate) concentration of HCl noted. Temperature, 37°C.

Approximate total concentration of HCl.	pH	$C_H \times 10^4$	Specific conductivity of (Reciprocal ohms $\times 10^4$.)			Time for gelatin to liquefy.	
			Solution.	HCl of same pH as solution.	Gelatin chloride ($= \kappa$ solution $- \kappa$HCl).	Hours.	Rate $= \frac{40}{\text{hrs.}}$
N							
0.02	4.23	0.60	17.2	0.27	17.0	4.5	9
0.04	3.50	3.16	33.1	1.47	31.6	1.1	36
0.06	2.78	16.6	48.2	7.7	40.5	0.40	100
0.08	1.78	166.0	110.0	76.0	34.0	0.42	95
0.10	1.48	331.0	175.0	151.0	24.0	0.65	62
0.12	1.26	550.0	260.0	245.0	15.0	0.92	43

EXPERIMENTAL.

50 gm. (dry weight) of purified isoelectric gelatin were dissolved in warm water and the volume was made up to 500 cc. Increasing amounts of HCl were then added to a series of 50 cc. portions of this solution and the volume of each portion was then made up to 100 cc. 2 cc. of 2 per cent pepsin solution were then added to 75 cc. of each of the above solutions and the solutions put in the water bath at 37°C. At short intervals 5 cc. samples were pipetted from each of the solutions into a series of test-tubes containing 2 cc. of water. These tubes were then placed in a water bath at 2°C. for 10 minutes, taken out, and the degree of liquefaction was compared with that of a standard tube. (This is a slight modification of the method of Fermi as described by Dernby.[27]) This procedure was repeated until a sample from each of the tubes showed the same degree of liquefaction as the standard tube. In this way the time necessary to produce a certain degree of liquefaction can be accurately and easily determined. The pH and con-

[27] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

ductivity of the solution were determined on the remaining 25 cc. of solution to which had been added the equivalent amount of inactivated pepsin. The determinations were made as described above except that the measurements were made at 37°C.

The Combination of Pepsin and Gelatin.

In a former paper[28] it was shown that the amount of pepsin which combined with a given quantity of coagulated egg albumin depended entirely on the reaction of the solution in which the egg albumin was suspended. The greatest amount of pepsin was combined when the

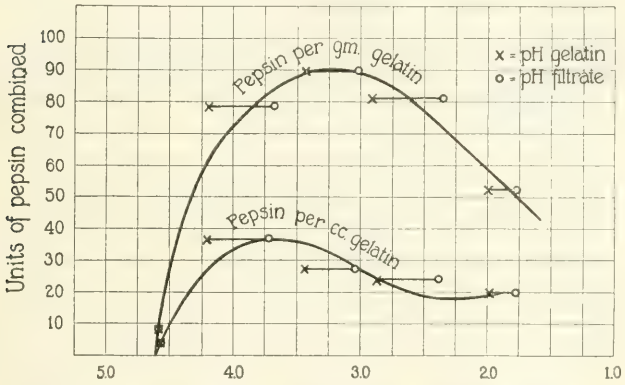

FIG. 4. Influence of pH on the combination of pepsin and gelatin.

solution had a reaction of pH 2.5 to 3.0. It was pointed out that this was probably part of the mechanism that caused insoluble proteins to digest more rapidly at this reaction than at any other since it seems that the rate of digestion must depend on the amount of pepsin in the solid protein.

These experiments have been repeated with gelatin and show in general the same result. The results of such an experiment are given in Fig. 4 and Table III. The figures show that a greater amount

[28] Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

of gelatin and pepsin is combined at about pH 3.0 than in either more or less acid solutions. In the case of gelatin the volume varies greatly with the reaction owing to the effect of the acid on the swelling of gelatin. The swelling is greatest at about pH 3.4 (*cf.* Loeb[20]). It might be supposed therefore that more pepsin was combined with the gelatin at about this degree of acidity simply because there was

TABLE III.

Combination of Gelatin and Pepsin.

5 gm. of isoelectric purified gelatin (= 0.75 gm. of dry weight) suspended in 200 cc. of HCl of strength noted and left 16 hours at 2°C. Filtered and washed twice with 100 cc. of water (5°C.) and total volume made up to 75 cc. 5 cc. of 2 per cent pepsin added. Allowed to stand 20 min. at 5° with occasional stirring. 4 cc. of supernatant fluid pipetted off and pepsin determined* in 1 cc. of this sample. Gelatin filtered and volume of filtrate measured. Gelatin melted and pH determined of this and of the filtrate.

Concentration of HCl.	pH of		Volume of filtrate.	Volume of gelatin (= 80 − volume of filtrate).	Units of pepsin.			
	Filtrate.	Gelatin.			Pepsin per cc. of filtrate.	Total pepsin in filtrate (*a*).	Total pepsin in gelatin (115-*a*).	Pepsin per 10 cc. of gelatin.
			cc.	*cc.*				
0	5.3	4.6	63	17	1.7	107.0	8	5
$\frac{M}{256}$	3.6	4.2	57	23	0.65	37.0	78	34
$\frac{M}{64}$	3.0	3.4	47	33	0.52	25.0	90	27
$\frac{M}{8}$	2.4	2.9	47	33	0.71	33.0	82	25
$\frac{M}{4}$	1.8	2.0	53	27	1.17	62.0	53	20
0 Control. No gelatin.	5.2		80	0	1.44	115.0	0	0

* *Cf.* Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113. The relative amount of pepsin is taken as the reciprocal of the time in hrs. required to cause a 5 per cent change in the conductivity of a 5 per cent egg albumin solution titrated to pH 1.7 with hydrochloric acid when 1 cc. of pepsin solution is added to 25 cc. of egg albumin at 37°C. The unit of pepsin is taken as that amount which when dissolved in 1 cc. and added to 25 cc. of the egg albumin solution will cause a change of 5 per cent in the conductivity in 1 hr.

a greater volume of gelatin present at this point. It will be seen, however, from Table II and Fig. 3 that this is not true since the figures show that there is a maximum even when the results are calculated to the basis of pepsin per cubic centimeter of gelatin. That is, there is not only more pepsin combined with a gram of gelatin at this pH but also the concentration of the pepsin in the gelatin is greatest here. There is considerable uncertainty as to the pH measurements since, as the table shows, the reaction of the liquid was always considerably more acid than that of the gelatin. In most of the experiments the difference was much more marked than in the experiment given; in some cases the maximum fell at about pH 2.2. This agrees much more closely with the optimum acidity found for digestion and for the ionization of the protein. Owing to the uncertainty of the pH measurement, however, it is probably better to make no definite statement as to the exact position of the optimum acidity for the combination between the gelatin and pepsin. The determining factor in regulating the amount of pepsin which is combined with the gelatin is the chemical condition of the gelatin and pepsin and not a difference in the rate of diffusion of the pepsin since the same curve is obtained irrespective of the time (after the first few minutes) during which the gelatin is left in the solution. The simplest explanation would seem to be that the gelatin combines only with the ionized protein and the amount combined therefore is dependent on the amount of ionized gelatin present. Pepsin therefore behaves just as do the inorganic anions studied by Loeb[20] as far as the influence of the hydrogen ion concentration on the combination is concerned.

DISCUSSION AND SUMMARY.

The experiments described above show that the rate of digestion and the conductivity of protein solutions are very closely parallel. If the isoelectric point of a protein is at a lower hydrogen ion concentration than that of another, the conductivity and also the rate of digestion of the first protein extends further to the alkaline side. The optimum hydrogen ion concentration for the rate of digestion and the degree of ionization (conductivity) of gelatin solutions is the same, and the curves for the ionization and rate of digestion as

plotted against the pH are nearly parallel throughout. The addition of a salt with the same anion as the acid to a solution of protein already containing the optimum amount of the acid has the same depressing effect on the digestion as has the addition of the equivalent amount of acid. These facts are in quantitative agreement with the hypothesis that the determining factor in the digestion of proteins by pepsin is the amount of ionized protein present in the solution. It was shown in a previous paper[11] that this would also account for the peculiar relation between the rate of digestion and the concentration of protein. The amount of ionized protein in the solution depends on the amount of salt formed between the protein (a weak base) and the acid. This quantity, in turn, according to the hydrolysis theory of the salts of weak bases and strong acids, is a function of the hydrogen ion concentration, up to the point at which all the protein is combined with the acid as a salt. This point is the optimum hydrogen ion concentration for digestion, since the solution now contains the maximum concentration of protein ions. The hydrogen ion concentration in this range therefore is merely a convenient indicator of the amount of ionized protein present in the solution and takes no active part in the hydrolysis. After sufficient acid has been added to combine with all the protein, *i.e.* at pH of about 2.0, the further addition of acid serves to depress the ionization of the protein salt by increasing the concentration of the common anion. The hydrogen ion concentration is, therefore, no longer an indicator of the amount of ionized protein present, since this quantity is now determined by the anion concentration. Hence on the acid side of the optimum the addition of the same concentration of anion should have the same influence on the rate of digestion irrespective of whether it is combined with hydrogen or some other ion (provided, of course, that there is no other secondary effect of the other ion). The proposed mechanism is very similar to that suggested by Stieglitz and his coworkers[29] for the hydrolysis of the imido esters.

Pekelharing and Ringer[5] have shown that pure pepsin in acid solution is always negatively charged; *i.e.*, it is an anion. The experiments described above show further that it behaves just as would be expected of any anion in the presence of a salt containing the protein ion as the cation and as has been shown by Loeb[20] to be the case with inorganic anions.

[29] Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 164, 402, 437, 586. 719

Nothing has been said in regard to the quantitative agreement between the increasing amounts of ionized protein found in the solution (as shown by the conductivity values) and the amount predicted by the hydrolysis theory of the formation of salts of weak bases and strong acids. There is little doubt that the values are in qualitative agreement with such a theory. In order to make a quantitative comparison, however, it would be necessary to know the ionization constant of the protein and of the protein salt and also the number of hydroxyl (or amino) groups in the protein molecule as well as the molecular weight of the protein. Since these values are not known with any degree of certainty there appears to be no value at present in attempting to apply the hydrolysis equations to the data obtained.

It it clear that the hypothesis as outlined above for the hydrolysis of proteins by pepsin cannot be extended directly to enzymes in general, since in many cases the substrate is not known to exist in an ionized condition at all. It is possible, however, that ionization is really present or that the equilibrium instead of being ionic is between two tautomeric forms of the substrate, only one of which is attacked by the enzyme. Furthermore, it is clear that even in the case of proteins there are difficulties in the way since the pepsin obtained from young animals, or a similar enzyme preparation from yeast or other microorganisms, is said to have a different optimum hydrogen ion concentration than that found for the pepsin used in these experiments. The activity of these enzyme preparations therefore would not be found to depend on the ionization of the protein. It is possible of course that the enzyme preparations mentioned may contain several proteolytic enzymes and that the action observed is a combination of the action of several enzymes. Dernby[27] has shown that this is a very probable explanation of the action of the autolytic enzymes. The optimum hydrogen ion concentration for the activity of the pepsin used in these experiments agrees very closely with that found by Ringer for pepsin prepared by him directly from gastric juice and very carefully purified. Ringer's pepsin probably represents as pure an enzyme preparation as it is possible to prepare. There is every reason to suppose therefore that the enzyme used in this work was not a mixture of several enzymes.

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RADIOACTIVITY AND PHYSIOLOGICAL ACTION OF POTASSIUM.

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I. INTRODUCTION.

The cations Na, K, and Ca are essential constituents of physiologically balanced salt solutions such as blood serum, tissue fluids, and sea water, and, in the absence of one of these ions, physiological processes cannot, as a rule, occur for any great period of time. Zwaardemaker[1] has recently advanced the interesting idea that the indispensability of potassium in cardiac action is due to the slight radioactivity of that element. To prove this idea, he has demonstrated that other radioactive substances, *e.g.* thorium, uranium, ionium, radium, etc., can replace the K ion in restoring heart beat after the heart has stopped beating in a Ringer solution containing no potassium. The most significant fact demonstrated by Zwaardemaker and his collaborators is that for the replacement of potassium by other radioactive substances, equiradioactive doses are required. Zwaardemaker's conclusion that the action of potassium in physiologically balanced salt solutions is a result of its radioactivity is of such great importance that it seemed justifiable to test the applicability of this view to physiological processes other than the heart beat.

As a result of experiments by Herbst[2] and others, it is known that sea urchin eggs are unable to develop when placed in potassium-free sea water immediately after fertilization, and Herbst found that rubidium and cesium could replace potassium to a limited extent. It seemed to us that a further study of the replacement of K by cesium

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273.

[2] Herbst, C., *Arch. Entwcklngsmechn. Organ.*, 1901, xi, 617.

and certain radioactive substances might help in deciding definitely whether or not the physiological influence of potassium is dependent on its radioactive properties.

II. EXPERIMENTAL.

The method used in this work was as follows. Eggs of *Arbacia* were fertilized in potassium-free artificial sea water of the following composition: 100 cc. of M/2 NaCl + 7.8 cc. of M/2 $MgCl_2$ + 3.8 cc. of M/2 $MgSO_4$ + 1.75 cc. of M/2 $CaCl_2$. To this solution enough M/10 $NaHCO_3$ (usually 0.2 cc. per 25 cc. of solution) was added to bring the pH to between 7.4 and 8.0. The chemicals used were Kahlbaum's purest preparations. Both glass- and metal-distilled water were used in preparing the solutions. After fertilization, the eggs were washed three times in similar K-free artificial sea water and were then placed in various solutions to observe development. In all experiments, some of the fertilized eggs were placed in (*a*) normal sea water, and (*b*) alkaline K-free sea water for control. In no case where eggs were placed in K-free sea water did the development go beyond the sixteen cell stage, and in a few hours the eggs were disintegrated. All sea water controls developed into normal plutei.

The criterion used in these experiments for the adequacy of the substitutes for potassium was the formation of normal swimming blastulæ within 24 hours. In reality development went beyond this stage in most instances.

A. Potassium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough KCl to make the total concentration of KCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., up to M/18, the latter concentration of KCl being about 5.5 times that present in normal sea water. The pH in these and succeeding experiments was about 7.4.

From Table I we see that fertilized eggs do not form swimming larvæ in artificial sea water with a concentration of KCl lower than M/660. We also see that a certain excess of potassium, *i.e.* 5.5 times its normal concentration in sea water, does not interfere with development up to the gastrula stage.

B. Rubidium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of K-free sea water to which was added enough RbCl to make the total concentration of RbCl in the dishes M/4,600, M/2,300, M/1,300, M/850, M/660, M/550, M/470, M/370, M/330, etc., to M/18.

The experiments proved that RbCl is entirely able to replace KCl in the development of swimming blastulæ and that the minimal concentration of RbCl needed is M/660, approximately that needed when KCl is used.

TABLE I.

Concentration of Potassium Needed for Development.

Molecular concentration of KCl.	Stage of development after	
	5 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	2–16 " "	"
M/2,300	2–16 " "	"
M/1,300	Few as far as 64 cells, mostly disintegrating.	"
M/850	" " " " 64 " " "	"
M/660	" " " " 64 " " "	Few swimming gastrulæ.
M/550	Some disintegrating, many good early blastulæ.	Swimming gastrulæ.
M/470	Almost all early blastulæ.	All gastrulæ.
M/370	" " " "	" "
M/330	All early blastulæ.	" "
M/150	" " "	" "
M/125	" " "	" "
M/100	" " "	" "
M/52	" " "	" "
M/27	" " "	" "
M/18	" " "	" "

C. Cesium Chloride Experiments.—Eggs were fertilized and washed as described, and were then placed in various dishes all containing 25 cc. of potassium-free sea water to which was added enough CsCl to make the total concentration of CsCl in the dishes M/4,600, M/2,300, M/1,300, M/1,000, M/850, M/660, M/500, M/250, M/125, etc., to M/18.

From Table II we see that CsCl can replace potassium chloride and that the minimal molecular concentration required for this purpose is practically identical for both salts. If the action of potassium is

due to its radioactivity, we should have to conclude that cesium has the same degree of radioactivity as potassium which is contrary to the facts known at present. Development with CsCl is possibly slower than with KCl, and the development does not go so far as in

TABLE II.

Concentration of Cesium Needed for Development.

Molecular concentration of CsCl.	Stage of development after	
	6 hrs.	24 hrs.
0	2–16 cells disintegrating.	Dead.
M/4,600	All disintegrating.	"
M/2,300	8–16 cells disintegrating.	"
M/1,300	8–16 " "	"
M/1,000	Few 16–32 cells, most disintegrating.	"
M/850	Few 64 cells, most disintegrating.	"
M/660	" 64 " " "	"
M/500	About 50 per cent in 64 cell stage.	About 50 per cent very slowly swimming blastulæ.
M/250	Most in 64 or 128 cell stage	About 50 per cent slowly swimming blastulæ.
M/170	Practically all 128 cells.	Still more swimming blastulæ.
M/125	" " 128 "	Mostly rapidly swimming blastulæ; few gastrulæ.
M/100	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/80	" " 128 "	Most blastulæ; some rapidly swimming gastrulæ.
M/64	" " 128 "	Same, but more gastrulæ.
M/44	" " 128 "	Swimming blastulæ, many degenerating.
M/33	" " 128 "	Mostly slowly swimming blastulæ.
M/18	" " 128 "	Mostly swimming gastrulæ.

the case of KCl probably on account of the greater toxicity of cesium which in rather low concentrations kills the larvæ.

D. Thorium Chloride Experiments.—It seemed important to determine whether or not potassium could be replaced by a radioactive element like thorium. Zwaardemaker found that in winter a solu-

tion of M/10,000 and in summer about M/100,000 thorium nitrate could replace potassium in his experiments on the heart. In our experiments, we fertilized eggs as described and after thorough washing in K-free sea water they were placed in dishes containing 25 cc. of K-free sea water and enough thorium chloride to make the total concentration of $ThCl_4$ in the dishes M/200,000, M/100,000, M/66,000, M/33,000, M/25,000, M/21,000, M/11,000, M/7,500, M/4,800, M/3,300, and M/2,100. To these solutions, which were quite acid, enough M/10 $NaHCO_3$ was added to bring the pH between 7.2 and 8.0. In all these

TABLE III.

Effect of Thorium in Replacing Potassium.

Molecular concentration of $ThCl_4$.	Stage of development after	
	4 hrs.	24 hrs.
0	2–8 cell stage disintegrating.	Dead.
M/200,000	2–8 " " "	"
M/100,000	2–8 " " "	"
M/66,000	2–8 " " "	"
M/33,000	2–8 " " "	"
M/25,000	2–8 " " "	"
M/21,000	2–8 " " "	"
M/11,000	2–8 " " "	"
M/7,500	2–8 " " "	"
M/4,800	2–8 " " "	"
M/3,300	2–8 " " "	"
M/2,100	2–8 " " "	"

cases the Th was probably in suspension and not in true solution and in the three highest concentrations of $ThCl_4$ addition of bicarbonate caused visible precipitation. Since Zwaardemaker's Ringer solutions were also slightly alkaline the Th in his solutions was also probably in suspension, but this would not interfere with the radioactivity since the radioactivity depends on the changes in the nucleus of an atom and since these nuclear changes do not depend on whether or not the substance is in true solution. As is seen in Table III in no case where Th was used to replace K did we observe the development of swimming blastulæ and in all dishes disintegration occurred before the sixteen cell stage was reached just as in the K-free sea water

control. It was shown that death in the thorium experiments was due to lack of K and not to the toxicity of $ThCl_4$, because when $ThCl_4$ was added to normal sea water in the same concentrations in which it was used to replace KCl it in no way interfered with the formation of normal larvæ, owing probably to the fact that the Th was in suspension and not in true solution. The radioactivity of the $ThCl_4$ was not sufficient to interfere with the development of the eggs up to the pluteus stage at least.

E. Uranium Acetate Experiments.—A series of experiments with the radioactive element uranium also gave negative results; *i.e.*, in no case were swimming larvæ obtained where uranium acetate was used to replace potassium and the eggs died before the sixteen cell stage was reached. The concentrations of uranium acetate used in the K-free sea water were M/125,000, M/62,500, M/42,000, M/32,000, M/25,000, M/16,000, M/12,500, M/11,000, M/6,800, M/4,700, M/3,000, and M/2,100. The pH in these solutions was brought to about 7.6 through the addition of $NaHCO_3$ and in the two highest concentrations some uranium was visibly precipitated. The lack of development of the eggs here was not due to the toxicity of the uranium salt because, when the uranium acetate was added to sea water in the same concentrations, normal larvæ developed except in the two highest concentrations and here the pH was found to be below 7. The uranium as well as the thorium was probably in suspension in the sea water and not in true solution but this, of course, did not influence the radioactivity. It is of interest to note that, here and in the thorium experiments, the radioactive elements did not inhibit the action of K as Zwaardemaker seems to assume.

III. Theoretical Remarks.

It appears from the foregoing experiments that in the development of the fertilized egg of *Arbacia* up to the blastula-gastrula stage potassium can be replaced by the non-radioactive Cs ion, the minimum concentration required for development being practically the same for both cations (KCl minimum concentration = M/660, CsCl minimum concentration = M/500). Furthermore, it is apparent that K cannot be replaced in our experiments by suspensions of the radioactive elements thorium and uranium. Hence, the action of potassium in the

development of the eggs of *Arbacia* cannot be attributed to its very slight radioactivity.

These results would seem at variance with the findings of Zwaardemaker and his coworkers in their experiments on the heart were it not for the fact that they also were able to replace potassium by cesium, which Zwaardemaker designates as "physiologically but not physically" radioactive. As a matter of fact nobody has thus far shown that Cs possesses more radioactivity than any other non-radioactive element; *e.g.*, Na. Zwaardemaker's observation that a heart which has ceased to beat in a solution lacking KCl can be resuscitated by radioactive substances may be explained without the assumption that K acts through its radioactivity. Lingle[3] found that when the ventricle of the heart of the turtle was suspended in a moist chamber where the air was replaced by pure oxygen the strip was able to beat for a considerable time, as long as 3 days, after the beats had been initiated by submersing the ventricle for a short time in a pure NaCl solution. The beats continued in some cases until the putrefaction of the heart tissues put a stop to them. This experiment proves that the KCl is not needed to provide the stimulus for the heart beat but that it is only needed to counteract some of the toxic effects of a pure NaCl solution when the heart is submersed in such a solution. Lingle found, moreover, that the heart which had stopped beating in a pure solution of NaCl began to beat again when 10 cc. of 3 per cent H_2O_2 were added to 90 per cent of the isotonic solution of NaCl. He could show by control experiments that only the bubbles of O_2 with which the muscle became frosted were responsible for the resuscitation. The ventricle thus resuscitated could then continue to beat for many hours. Lingle suggests that the oxygen supply in a solution is inadequate for a heart beating in a pure solution of NaCl unless a more rapid source of supplying oxygen than mere diffusion from the air is provided. We do not know how the increased supply of oxygen can resuscitate the heart which had ceased to beat in a pure solution of NaCl but the tentative suggestion may be permitted that this effect is due to the transformation of a harmful substance formed during the activity of the heart in the absence of K, or Ca, or

[3] Lingle, D. J., *Am. J. Physiol.*, 1902–03, viii, 75.

both, into a less harmful one through a more rapid oxidation. Lind has shown that penetrating rays can cause the formation of water from O and H and that H_2O_2 seems to be an intermediate product in the reaction.[4] On the basis of these observations it is quite probable that H_2O_2 is formed or that oxygen is activated in some other form when penetrating rays go through the cells of the heart, and that the rate of oxidation is increased in the cells of the heart. This might explain Zwaardemaker's experiments on the restoration of the heart beat by U, Th, and radium without compelling us to assume that KCl, Rb, and even Cs act physiologically by radioactivity.

CONCLUSIONS.

1. The non-radioactive cesium ion can replace the potassium ion almost quantitatively in solutions required for the development of the egg of the sea urchin into swimming blastulæ.

2. Thorium chloride and uranium acetate cannot replace the potassium chloride in the solutions required for the development of the egg.

3. Thorium chloride and uranium acetate do not antagonize the action of the potassium contained in sea water upon the development of eggs.

[4] Lind, S. C., *J. Am. Chem. Soc.*, 1919, xli, 551.

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CHEMICAL CHARACTER AND PHYSIOLOGICAL ACTION OF THE POTASSIUM ION.

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I. INTRODUCTION.

Zwaardemaker[1] has recently made the interesting suggestion that the rôle of potassium in physiologically balanced salt solutions—*e.g.* the blood or the sea water—is due to the very slight radioactivity of this element and not to its chemical character as determined by its position in the series of elements. It has been pointed out by R. F. Loeb[2] in another paper in this *Journal* that K cannot be replaced by Th and U as far as the development of the sea urchin egg is concerned, that the non-radioactive element Cs is capable of replacing potassium to some extent in this case, and that Zwaardemaker's observations on the influence of radioactive substances on the heart beat might be explained without the assumption that the physiological action of K is due to its radioactivity.

This then suggests that the physiological action of potassium is due to its chemical character. We know through the work of Sir Ernest Rutherford that radioactivity is caused by an explosive change in the nucleus of the atom while the chemical and most of the physical properties of the atom depend upon its external ring or shell of electrons. These latter properties are repeated periodically in the series of elements arranged by their atomic numbers and if we can show that the physiological action of an element corresponds to its position in the periodic table we know that we are dealing with purely chemical effects and not with radioactive effects. We intend to show in an indirect way that the action of K in physiologically balanced salt

[1] Zwaardemaker, H., *J. Physiol.*, 1919–20, liii, 273

[2] Loeb, R. F., *J. Gen. Physiol.*, 1920–21, iii, 229.

solutions corresponds to its purely chemical character; *i.e.*, its position in the periodic table (or rather to its atomic number and arrangement of external electrons).

II. The Resemblance in the Antagonistic Effects of the NH_4 and K Ions.

According to their physiological action the ions of the alkaline metals can be arranged in two distinct groups, the one including Li and Na, the other K, Rb, and Cs. The difference in the two groups is noticeable in various phenomena. Isotonic solutions of LiCl and NaCl will give rise to muscular twitchings while KCl, RbCl, and probably CsCl will not. Experiments on the egg of *Fundulus* show that toxic solutions of salts with bivalent metals, such as $MgCl_2$ or $CaCl_2$, can be rendered less toxic by the addition of KCl, RbCl, or CsCl, but not or practically not by the addition of NaCl or LiCl.[3] It is known that the NH_4 ion resembles in its chemical behavior the K ion more closely than it does the sodium ion, and Langmuir[4] has utilized this fact in support of his theory of the cubical atom. If it be true that the physiological action of K depends upon its chemical character, the close resemblance between the chemical character of NH_4 and K should express itself in phenomena of antagonism. NH_4Cl is generally very toxic for cells but it can be used with good effect in experiments on the egg of *Fundulus* which is surrounded by a rather impermeable membrane. In experiments on *Fundulus* it can be shown that the antagonistic action of the NH_4 ion is like that of the members of the K group and not like that of the members of the Na group.

When newly fertilized eggs of *Fundulus* are put into a 5 M/32 solution of $CaCl_2$ practically no egg (*i.e.* less than 2 per cent of the eggs) can form an embryo. When the 5 M/32 $CaCl_2$ solution is made up in solutions of different chlorides instead of in H_2O it is found that in LiCl and NaCl the toxicity of the $CaCl_2$ solution is not diminished. When, however, the 5 M/32 $CaCl_2$ solution is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable percentage of the eggs can develop into embryos as is shown in Table I.

[3] Loeb, J., *J. Biol. Chem.*, 1914, xix, 431.
[4] Langmuir, I., *J. Am. Chem. Soc.*, 1920, xlii, 274.

The same fact can be demonstrated equally well with other toxic solutions; *e.g.*, Na_3 citrate. If newly fertilized eggs of *Fundulus* are put into M/100 Na_3 citrate practically no egg can form an embryo, and the result remains the same if the M/100 solution of Na_3 citrate is made up in different concentrations of LiCl or NaCl. When, however, the M/100 solution of Na_3 citrate is made up in KCl, RbCl, CsCl, or NH_4Cl a considerable number of eggs develop into embryos as indicated in Table II.

TABLE I.

	Percentage of newly fertilized *Fundulus* eggs which can form embryos in 5 M/32 $CaCl_2$ when this solution is made up in						
	0	M/80	M/40	M/20	M/10	M/5	3 M/10
LiC	1.5	0	0	0	0	0	0
NaCl		1	2	1	0	0	0
KCl		5	21	21	44	60	64
RbCl		19	23	26	40	54	43
CsCl		3	4	14	9	17	30
NH_4Cl		1	0	3	4	17	16

TABLE II.

	Percentage of eggs of *Fundulus* which can develop in M/100 sodium citrate solution if this solution is made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
LiCl	0	3	2	0	0	0
NaCl	0	6	0	2	1	0
KCl	0	26	19	57	52	53
RbCl	8	46	55	60	55	42
CsCl	8	40	60	56	32	
NH_4Cl	8	2	1	2	45	36

The table shows that when the M/100 Na_3 citrate solution is made up in M/5 KCl, RbCl, CsCl, or NH_4Cl practically half the eggs form embryos. In this experiment the addition of the Cl ion may diminish the toxicity of the citrate solution but if this be true the fact remains that the Cl ion can have this effect only when it is added with K or NH_4 ions and not when added with Na or Li ions.

The NH_4 ion, therefore, resembles in its physiological behavior the K ion more than it does the Na or Li ion.

III. The Antagonism between Li and K.

In any series of ions based on their chemical or physical behavior Na occupies a position between Li and K. Li has a smaller and K has a larger ionic radius than Na. If we replace some of the Na ions of sea water by Li ions we alter the properties of the solution in one sense, and if we replace part of the Na ions by K ions we alter the properties in the opposite sense. We should, therefore, expect that if we replace a certain percentage of Na ions in sea water by Li ions the toxic character of the solution should be diminished if we replace at the same time also a certain percentage of the Na ions by K ions; since with the combined increase of the K ions and of Li ions the effect of Na ions might be more nearly approximated.

The newly fertilized egg of the sea urchin (*Arbacia*) can develop into gastrulæ in an "artificial sea water" of the following composition.

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
2.2 cc. of M/2 KCl
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$

To this was added 0.8 cc. of M/10 $NaHCO_3$ to bring the artificial sea water to a pH of about 7.4.

We prepared the following solution in which M/2 NaCl of the artificial sea water was replaced by M/2 LiCl and which was free from K. Its composition was:

100.0 cc. of M/2 LiCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

This solution, which we will call the Li mixture, permitted us to replace the Na in natural or artificial sea water by Li without altering the constitution of the sea water in any other direction except in regard to K, the concentration of which it was our intention to vary in the experiments.

Our first experiments consisted in mixing various quantities of natural sea water and Li mixture to find out the maximal amount of Li in natural sea water which still permitted the formation of normal blastulæ in about 16 or 20 hours; the eggs were put into the solution immediately after fertilization. It was found that only 8 per cent of the Li mixture could replace the natural sea water without preventing the development of the eggs into swimming blastulæ. When, however, the proportion of KCl contained in the sea water was increased thirteen times its normal amount the eggs were able to develop into larvæ when as much as 52 per cent of Na was replaced by Li. It is therefore possible to increase the tolerance of the sea urchin egg

TABLE III.

Maximal Amount of Li in which Swimming Blastulæ of Arbacia Can Be Obtained.

K mixture.	Natural sea water.	Li mixture.
cc.	*cc.*	*cc.*
0.0	23.0	2.0
0.5	19.5	5.0
1.0	18.0	6.0
2.0	16.0	7.0
4.0	12.0	9.0
6.0	6.0	13.0

against Li 600 per cent by increasing simultaneously the amount of K normally present in the sea water by 1,300 per cent. Since it was necessary to keep all the other constituents of the sea water constant the KCl was not added in the form of a pure M/2 solution of this salt but in the form of a mixture of the following composition which we will call the KCl mixture.

100.0 cc. of M/2 KCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $KHCO_3$

Systematic experiments showed that the maximum dose of Li in which the eggs could develop into larvæ increased with the concentration of K added to the sea water. This is indicated in Table III.

The table shows that if we wish to replace in normal sea water more Na by Li we must at the same time also replace an increasing proportion of Na by K.

In order to obtain a more regular curve than expressed in Table III we replaced the natural sea water by a NaCl mixture free from KCl and made up as follows:

100.0 cc. of M/2 NaCl
1.75 cc. of M/2 $CaCl_2$
7.8 cc. of M/2 $MgCl_2$
3.8 cc. of M/2 $MgSO_4$
0.8 cc. of M/10 $NaHCO_3$

Experiments were then made to ascertain the maximal amount of Li mixture which permitted the formation of swimming blastulæ for each given amount of KCl. The results are contained in Table IV.

TABLE IV.

Maximal Amount of Li Mixture Permitting Formation of Swimming Blastulæ.

K mixture.	Na mixture.	Li mixture.
cc.	*cc.*	*cc.*
0.1	24.0	1.0
0.2	23.0	2.0
0.5	22.0	3.0
1.0	20.0	4.0
2.0	17.0	6.0
4.0	13.0	8.0
6.0	10.0	9.0
7.0	8.0	10.0

When 8 cc. of K mixture were used no more larvæ were obtained on account of the fact that this concentration of K itself was too toxic.

Table IV shows more clearly than Table III that by replacing more Na ions by K ions we increase at the same time the proportion of Na ions which can be replaced by Li ions, without preventing the development of the sea urchin egg into a swimming larva.

It was then shown that Rb has a similar but not quite so great an effect as K (Table V). RbCl was also added in the form of a mixture containing all the other constituents of sea water except K and Na.

Cs acts also somewhat like K but still less weakly than Rb. Only one series of experiments was made in an attempt to replace K by Cs, and this series proved that in 2 cc. of M/2 CsCl mixture + 19 cc. of natural sea water + 4 cc. of M/2 Li mixture swimming larvæ could be obtained. When no sea water was replaced by CsCl, 2 cc. of the Li mixture in 25 cc. was the maximum which still permitted the development of larvæ.

These results show that the toxic effects of Li, which occupies in the periodic table a position on one side of NaCl, are mitigated by the addition of ions like K, Rb, and Cs, occupying a position on the other side of Na. A mixture of Li and K ions in proper proportions acts more like a solution of Na ions than do the Li ions alone.

TABLE V.

Maximal Amount of Li Mixture Permitting Formation of Swimming Larvæ.

Rb mixture.	Sea water.	Li mixture.
cc.	cc.	cc.
1.0	20.0	4.0
2.0	18.0	5.0
4.0	14.0	7.0

It should be taken into consideration that in these experiments the balance between monovalent and bivalent cations was not disturbed.

If we replace a smaller or larger percentage of the Na ions contained in sea water by Mg or by Ca ions the toxicity of LiCl is not diminished. This is probably due to the fact that the quantity of Ca or Mg required for balancing the monovalent cations is naturally present in the sea water.

The antagonism between LiCl and KCl can also be demonstrated in the eggs of *Fundulus*. When newly fertilized eggs of *Fundulus* are put into M/5 LiCl all the eggs are dead before an embryo is formed. If, however, the M/5 solution of LiCl also contains a small quantity of RbCl or of KCl, as many as 20 per cent of the eggs live long enough to form embryos. When, however, the M/5 LiCl solution contains NaCl not a single embryo is formed. CsCl also gives a positive effect. The M/5 LiCl solution was made up in distilled water and in different

concentrations of the salts mentioned. Table VI gives the results of an experiment. The first horizontal row gives the molecular concentration of NaCl, KCl, RbCl, and CsCl in which the M/5 solution of LiCl was made up. The figures in the next horizontal rows give the percentage of eggs which formed embryos.

The table shows that the addition of NaCl did not protect the *Fundulus* eggs against the toxic effects of LiCl, while the KCl, RbCl, and CsCl had a protective or antagonistic effect. The protective effect of these salts is considerably less than that produced by a salt with a bivalent cation since in the latter case 80 per cent or more of the eggs form embryos in a M/5 solution of LiCl.

TABLE VI.

	Percentage of eggs which formed embryos in M/5 LiCl made up in					
	0	M/40	M/20	M/10	M/5	3 M/10
NaCl	0	0	0	0	0	0
KCl	0	18	10	14	11	6
RbCl	0	7	13	18	20	2
CsCl	0	2	0	5	9	2

These examples may suffice to show that the action of the potassium ion in the phenomena of antagonism (which underlie the physiological balance of ions in salt solutions) is in agreement with the purely chemical character of this ion, *i.e.* its position in the periodic table; and that hence there is no reason to attribute its physiological action in these cases to some other factor; *e.g.*, its extremely minute radioactivity.

SUMMARY.

1. It is shown that the NH_4 ion acts in cases of antagonism on the egg of *Fundulus* more like the K ion than the Na ion; this corresponds to the fact that in its general chemical behavior the NH_4 ion resembles the K ion more closely than the Na ion.

2. It is shown that the tolerance of sea urchin eggs towards the Li ion can be increased 500 per cent or more if at the same time a certain amount of Na ion is replaced by K, Rb, or Cs ions. Since in the

periodic table Na occupies a position between K and Li it is inferred that the Li and K ions deviate in their physiological action in the opposite direction from the Na ion.

3. These data indicate that the behavior of the K ion in antagonistic salt action (which forms the basis of the physiologically balanced action of ions) is due to its purely chemical character, *i.e.* its position in the periodic table or rather to its atomic number, and not to those explosions in its nucleus which give rise to a trace of radioactivity.

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ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. II.

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I. Combining Ratios of Acids and Bases with Gelatin and the Swelling of Gelatin.

In this paper we will continue the demonstration of the relation between the combining ratios of acids and bases with proteins and the effect of ions on the physical properties of proteins. This demonstration completes the proof that the purely chemical forces of primary valency determine the reactions of proteins with other compounds.

It is generally stated in colloidal literature that gelatin swells more in chlorides, bromides, or nitrates than in water and that it swells less in citrates, acetates, tartrates, phosphates, and sulfates than in water. The author of this statement is Hofmeister[1] who was a pioneer in this work and who cannot be blamed for not having considered certain sources of error in his methods. In Hofmeister's experiments gelatin blocks were put into salt solutions of so high a concentration that—as we now know—no specific ion effects could be expected and the slight differences in swelling actually observed by him were probably merely accidental. He even mentions that sugar solutions have a "dehydrating" effect, and this fact alone should have warned chemists against using his experiments for conclusions concerning the specific effects of ions on the physical properties of colloids. As far as the writer can determine from the literature the discrimination between "hydrating" and "dehydrating" ions originated from these experiments.

[1] Hofmeister, F., *Arch. exp. Path. u. Pharmakol.*, 1891, xxviii, 210.

It is often asserted that Hofmeister's ion series for swelling has been confirmed by other authors. Thus Zsigmondy[2] makes the following statements in support of this impression.

"Wo. Ostwald who compared the efficiency of different acids found that swelling diminishes in the acids in the following order,

$$HCl > HNO_3 > \text{acetic acid} > \text{sulfuric acid} > \text{boric acid.}$$

Fischer has shown that the acid and alkali swelling of gelatin as well as that of fibrin is diminished by the addition of salt, and that chlorides, bromides, and nitrates have a less dehydrating action than acetates, sulfates, or citrates. We have here a similar series as in the case of the precipitation of proteins by alkali salts, although the order does not agree entirely."

The writer is inclined to interpret Ostwald's and Fischer's experiments differently from Zsigmondy, since both authors ignored the hydrogen ion concentration of their solutions. We believe to have shown that it is necessary to base conclusions concerning the relative efficiency of ions on experiments with equal hydrogen ion concentration. By ignoring this postulate Ostwald has only succeeded in proving that acetic and boric are weaker acids than nitric but not that gelatin swells less in acetates or borates than in nitrates; and Fischer has only succeeded in proving that citrates and acetates are buffer salts which when added to a solution of a strong acid diminish its hydrogen ion concentration, but not that acetates and citrates diminish the swelling of gelatin. These authors attributed the effects caused by a variation in the hydrogen ion concentration of their solutions erroneously to an influence of the anion. The Hofmeister series of ion effects on swelling has,in reality, never been confirmed.

If we wish to study the specific effects of ions on the swelling of gelatin we must proceed from isoelectric gelatin, bring it to different pH values by different acids or alkalies, and then compare the swelling at the same pH for these different acids or alkalies. If this is done it is found that when gelatin is in combination with the anion of a weak dibasic or tribasic acid, *e.g.* tartaric, citric, phosphoric, its degree of swelling is practically the same as when it is in combination with Cl or NO_3; since in all these cases the anion of the gelatin salts is monovalent. Only in the case of gelatin sulfate is the swelling considerably less,

[2] Zsigmondy, R., Kolloidchemie, Leipsic, 2nd edition, 1918, 373.

because the anion is divalent, H_2SO_4 combining with gelatin in equivalent and not in molecular proportions as do the weak dibasic or tribasic acids; *e.g.*, tartaric or phosphoric.

A few words are necessary concerning the method of these experiments. We can measure the amount of swelling by determining the increase in weight of a given mass of gelatin or by determining its increase in volume. We have adopted the following simple and quick volumetric method (although we intend to supplement these experiments later with gravimetric experiments).

Dry powdered gelatin, of pH = 7.0, was sifted and the grains no longer going through Sieve 50 but going through Sieve 40 or 30 were selected for the experiment. We had therefore fairly uniform grains of not too small a diameter. Doses of 1 gm. each of such powder were weighed out, each dose was put for an hour into 100 cc. of M/128 acetic acid at 10°C. to bring the gelatin to the isoelectric point. The powdered mass was then put on a filter and washed five times with 25 cc. of distilled water at 5°C. In the acetic acid solution and during the washing on the filter the powdered gelatin is stirred constantly.

Each dose of originally 1 gm. of dry powder which had meanwhile absorbed a certain quantity of liquid (which was about the same for each gram of the isoelectric powder) was then put for 1 hour at about 20° into 100 cc. of different concentrations of the acid or base whose influence on swelling was to be tested, and the suspension was constantly agitated. It was found that in an hour the granules of gelatin had reached the maximal swelling in each solution. To measure the relative amount of swelling in different acids or alkalies and at different pH the suspension was poured into graduate cylinders of 100 cc. each (and all of the same diameter) in which the granules fell very rapidly to the bottom. The cylinders were kept in a water bath at 20°C. for about 10 to 15 minutes and the volume occupied by the gelatin granules was then read. This volume included a certain amount of solution between the granules and therefore the real volume of the gelatin was smaller than that read. While therefore the method cannot be used to measure the absolute amount of swelling it allowed us to determine the relative influence of different acids or bases on the swelling for the same pH.

The determination of the pH of the gelatin in these experiments requires a short discussion since the pH inside the gelatin is quite different from that in the supernatant liquid, owing to the Donnan equilibrium. Donnan has shown that when a solution of a colloidal salt is separated from the solution of a crystalloidal salt with a common ion by a membrane which is permeable for the crystalloidal but not for the colloidal ions the concentration of the crystalloidal salt is, at the point of equilibrium, always lower on the side of the colloidal solution than on the side of the crystalloidal solution.[3] This was invariably the case in our experiments on osmotic pressure reported in the preceding paper. When, for example, a gelatin chloride solution of pH 3.5 was put inside a collodion bag and the latter was dipped into a solution of HCl (without gelatin) also of a pH 3.5, the pH on the two sides of the membrane did not remain the same since some of the free acid was forced from the colloidal solution into the pure acid solution outside the collodion bag, so that the pH of the outside solution fell while that of the inside rose.

As Procter[4] has pointed out this Donnan equilibrium must play a rôle also in the case of the swelling of gelatin since in this case the surface of the gelatin granule takes the place of the membrane permeable for the crystalloidal electrolyte but not for the colloid.

In our experiments 1 gm. of originally isoelectric gelatin was put for 1 hour at 20°C. into 100 cc. of acid, *e.g.* HCl, of different concentration varying from M/16 to M/8,192. After an hour equilibrium was reached and the pH of the supernatant fluid was determined. The gelatin was put on a filter (after the volume of the gelatin in the graduate cylinder had been measured) and all the acid was allowed to drain off. A trace of outside acid probably remained on the surface of each granule though presumably some of the free acid inside the granule diffused to the surface under the influence of pressure. This error was partly but not completely compensated by adding enough distilled water of pH of about 5.6 to the gelatin after it had

[3] Donnan, F. G., *Z. Electrochem.*, 1911, xvii, 572. Donnan, F. G., and Harris, A. B., *J. Chem. Soc.*, 1911, xcix, 1554. Donnan, F. G., and Garner, W. E., *J. Chem. Soc.*, 1919, cxv, 1313.

[4] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

been melted to bring the volume to 100 cc. The pH of the 1 per cent solution of originally isoelectric gelatin was determined colorimetrically. It was found that the pH of the supernatant HCl solution was always considerably smaller than the pH of the gelatin solution (Table I).

The first row in Table I gives the molecular concentration of the 100 cc. of HCl into which the gelatin was originally put. The second row gives the pH of these supernatant HCl solutions after 1 hour, and the third row gives the pH of the gelatin solutions after the supernatant HCl solution had been drained off and after the remaining mass of gelatin had been melted and brought to a volume of 100 cc. by adding enough distilled water of pH 5.6. It will be noticed: first, that the pH of the supernatant HCl solution after 1 hour is higher than the pH of the original HCl solution owing to the fact that some acid combined with the gelatin; and, second, that the pH of the gelatin solution is considerably higher than that of the supernatant solution owing to the Donnan equilibrium, according to which the concentration of free acid outside the gelatin must be greater than inside.

In order to get the correct difference due to the Donnan equilibrium, solutions of gelatin salts were put into collodion bags and these bags were dipped into beakers containing350 cc. of solution of the same acid or base as that inside the collodion bag and possessing the same pH as the gelatin solution; the outside solution, of course, was free from gelatin. In the case of gelatin-acid salts free acid invariably diffused from the gelatin solution into the pure acid solution, *e.g.* HCl, so that the pH of the latter became smaller and that in the gelatin solution higher. In these experiments the volume of the outside pure HCl solution was 350 cc. and that of the inside 1 per cent gelatin solution only 50 cc. Table II gives the result of one experiment of this kind.

Thus at equilibrium pH the gelatin solution was 3.8 and the outside solution 3.2, or inside 4.0, and outside 3.3. This difference is in the same sense and of nearly but not quite the same order of magnitude as that observed in Table I.

We may therefore conclude that the real pH of the gelatin solution inside the granules of gelatin was slightly less than that measured by our method.

TABLE I.

Original concentration of supernatant HCl solution	M/16	M/32	M/64	3 M/256	M/128	3 M/512	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant HCl solution after 1 hr.	1.25	1.6	1.9	2.1	2.35	2.55	2.8	3.2	3.4	3.6	3.85	4.0
pH of 1 per cent gelatin solution after 1 hr.	2.2	2.3	2.7	3.0	3.25	3.4	3.8	4.2	4.6	4.65	4.7	4.7

TABLE II.

pH at beginning of experiment, inside and outside	2.3	2.6	3.0	3.1	3.3	3.5	3.6	3.75	4.1	4.38	4.8
pH of outside HCl solution after 20 hrs.	2.1	2.5	2.9	3.0	3.15	3.2	3.3	3.4	3.7	4.0	4.8
pH of gelatin solution after 20 hrs.	2.6	3.2	3.3	3.5	3.7	3.8	4.0	4.1	4.2	4.4	4.8

TABLE III.

Original concentration of supernatant KOH solution	M/32	M/64	M/128	M/256	M/512	7 M/4,096	6 M/4,096	5 M/4,096	M/1,024	M/2,048	M/4,096	M/8,192
pH of the supernatant KOH solution after 1 hr.	12.4	12.0	11.6	11.3	8.0	7.2	7.0	6.6	6.6	5.8	5.1	5.2
pH of 1 per cent gelatin solution after 1 hr.	12.0	11.6	11.0	9.8	6.3	5.7	5.5	5.4	5.3	5.1	4.9	4.8

The results of our experiments on swelling are expressed in Figs. 1, 2, and 3. The abscissæ in Fig. 1 are the pH found in the gelatin after equilibrium was established. The ordinates represent the figures for the volume of the granules of 1 gm. of gelatin in different acids. It is obvious that in all cases the volume (or swelling) is a

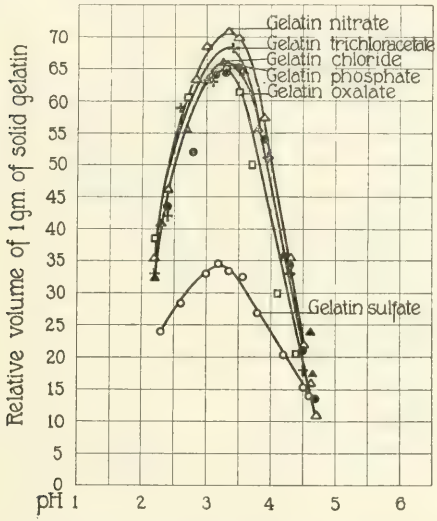

FIG. 1. Influence of HCl, HNO_3, H_3PO_4, H_2SO_4, trichloracetic, and oxalic acids on the swelling of gelatin. Abscissæ are the pH, ordinates the volume of gelatin. The curves for all the acids are practically identical except that for H_2SO_4 which is about one-half as high as the curves for the other acids.

minimum at the isoelectric point pH = 4.7, that it rises with diminishing pH until the maximum is reached at a pH of about 3.2 or 3.3, and that the curve drops steeply with a further diminution of pH (*i.e.* a further increase of hydrogen ion concentration). The fact that the maximum lies here at pH of about 3.2, while in our osmotic pressure curves it was at about 3.3 or 3.4, indicates the degree of error in

the measurement of pH in this case due to the adhesion of some of the original acid on the outside of the granules. This error was partly compensated by the addition of distilled water of pH of about 5.6 in making up the 1 per cent solution of gelatin. On the whole the

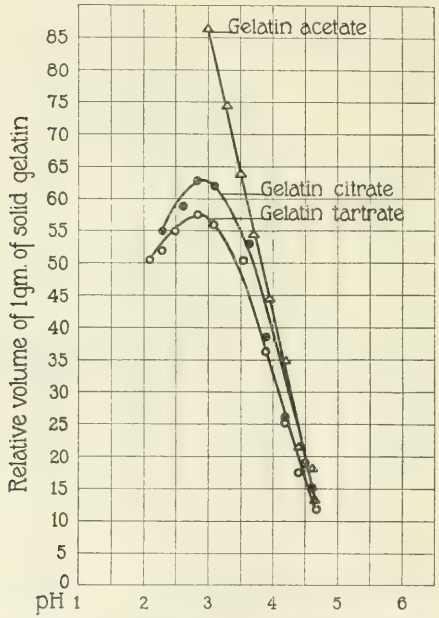

FIG. 2. Influence of citric, tartaric, and acetic acids on swelling of gelatin. The curves for citric and tartaric acids are practically identical with those for HCl and HNO_3 in Fig. 1. That for acetic acid is a little higher owing probably to some specific and secondary effect of this acid.

probable error was +0.1 or +0.2; *i.e.*, the real pH was 0.1 or at the utmost 0.2 greater than in our abscissæ. The most important fact is, however, that the curves for the influence of HCl, HNO_3, trichloracetic, oxalic, phosphoric, citric, and tartaric acids are practically

identical (Figs. 1 and 2), proving that only the effect of the valency and not that of the nature of the anion of the acid used influences the swelling; since we have seen that the anion of weak dibasic or tribasic organic acids combining with the gelatin is always monovalent.

The curve for the swelling of gelatin sulfate, where the anion combining with gelatin is bivalent, is only half as high as the curve for the salts of gelatin with the anion of weak dibasic acids (Figs. 1 and 2).

Acetic acid gives an increasing amount of swelling (Fig. 2), but it must be remembered that M/1 acetic acid had to be used to bring the pH of the gelatin to 3.0, and it is not impossible that in this case a secondary chemical or physical modification of the gelatin may complicate the conditions.

It is of interest to compare these curves with those which should be expected according to the Hofmeister series. In the latter case the curves for phosphate, oxalate, citrate, tartrate, and acetate should coincide with the curve for sulfate instead of coinciding with the curves for Cl and NO_3. This difference is due to the fact that the believers in the Hofmeister series did not determine the pH and that they erroneously ascribed the effects due to a variation in the hydrogen ion concentration to a difference in the influence of the anion.

The ratio between the effects of sulfuric acid on swelling and that of the other acids is again not far from 1:2. If we deduct the swelling of the isoelectric gelatin (of about 10 mm.) from the values of our ordinates the swelling of gelatin sulfate at pH of about 3.3 is less than one-half that of the other gelatin-acid salts where the anion in combination with gelatin is monovalent.

When powdered isoelectric gelatin is treated with an alkali, *e.g.* KOH, the supernatant watery solution is less acid or more alkaline than the gelatin granules. The CO_2 of the air lowers the pH of the solutions a little but this error affects the pH of the supernatant watery solution more than it does the gelatin which has a buffer action. Table III, p. 252, gives the original concentration of the watery solution of KOH, into 100 cc. of which 1 gm. of powdered isoelectric gelatin was put (first row, Table III). After 1 hour the pH of the supernatant watery solution was determined (second row, Table III);

the supernatant solution was drained off from the gelatin, the latter melted, and the volume brought to 100 cc. by adding distilled water of pH of about 5.6, and the pH of the gelatin solution was determined (third row, Table III).

It is obvious that the pH of the supernatant solution is higher than that of the gelatin solution, as we should expect from the Donnan equilibrium.

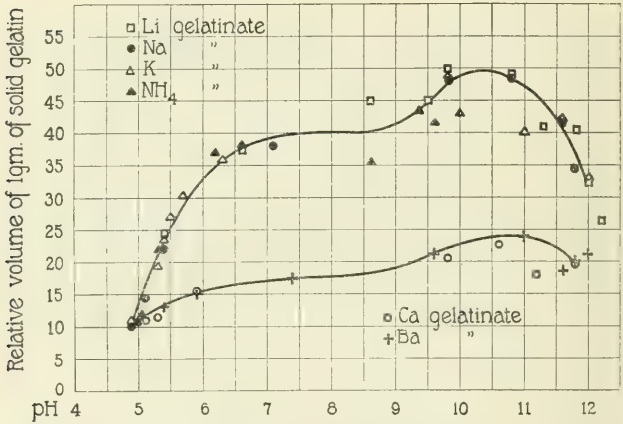

FIG. 3. Curves for the effect of different bases on swelling. Those for LiOH, NaOH, KOH, and NH_4OH are practically identical and about twice as high as those for $Ca(OH)_2$ and $Ba(OH)_2$.

Fig. 3 gives the curves for the action of alkalies on swelling. The curves for Li, Na, K, and NH_4 gelatinate of the same pH are practically identical, except that the values for NH_4OH are irregular for pH above 8.5 possibly on account of the fact that the concentration of NH_4OH required to bring gelatin to such pH is rather high. The main fact is that the ratio of the maximal swelling of gelatin salts with bivalent cation like Ca or Ba is half or possibly a little less than half of that of gelatin salts with monovalent cation, like Na, K, or NH_4.

Near pH = 7.0 the curves run parallel to the axis of abscissæ for the reason that a considerable variation in pH signifies only a negligible change in the concentration of gelatin salt formed. The experiments were not carried beyond a pH of 12.0 on account of the lack of reliable indicators for that region, and on account of the fact that alkali causes chemical changes in the gelatin.

It should be pointed out that the maximal swelling of gelatin in alkalies was less than that in acids. This was not observed in the osmotic pressure curves.

II. Relative Solubility of Different Gelatin Salts in Mixtures of Water and Alcohol.

When powdered gelatin is brought to the isoelectric point, melted, and made into a 1 per cent solution it is at first transparent. After some time, which is the shorter the lower the temperature, the gelatin solution becomes opaque; and in the course of weeks or months it may settle in the form of a precipitate. This, however, does not happen in each case, possibly for the reason that the precipitation will occur only at a very definite pH, while, with a slight deviation from this point in either direction, the result will be only an opacity at room temperature. Raising of the temperature will again result in the clearing of the opacity. The opacity seems therefore to be due to the formation of larger aggregates of protein molecules and these will float as long as they are not too large. The setting of the solution to a gel is a different process from this precipitation since no cloudiness or opacity needs to be connected with this latter phenomenon.

When we add to a freshly prepared solution of isoelectric gelatin only a trace of 95 per cent alcohol the cloudiness which would have formed slowly is noticed at once and if we add a little more alcohol we can produce at once a dense precipitate. In order to standardize the degree of cloudiness produced we add so much 95 per cent alcohol to 10 cc. of a 1 per cent solution of isoelectric gelatin in a test-tube of definite diameter until certain letters become illegible when looked at through the test-tube filled with the gelatin-alcohol-water mixture. Since the addition of alcohol to the watery solution raises the temperature and since this has the tendency to diminish the degree of

opacity of the mixture it was necessary to dip the test-tube in ice water during the process of mixing and keep the gelatin solution approximately at 10°C.

When we prepare gelatin chloride by adding small quantities of HCl to isoelectric gelatin we need the more alcohol the lower the pH and very soon a limit is reached when the addition of 25 cc. or more alcohol no longer brings about any precipitate or even cloudiness. Thus 10 cc. of isoelectric gelatin required 2 cc. of 95 per cent alcohol to bring about that high degree of opacity at which the test letters were no longer legible. When the pH of the gelatin was lowered to 4.55

TABLE IV.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of gelatin-acid salt.									
	4.6	4.55	4.45	4.4	4.2	3.75	3.3	3.1	2.6	2.1
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Gelatin chloride		5.5	∞	∞	∞	∞	∞	∞	∞	∞
" oxalate	5.3	18.1		∞	∞	∞	∞	∞	∞	∞
" phosphate		28.0		∞	∞	∞	∞	∞	∞	∞
" tartrate		11.6		∞	∞	∞	∞	∞	∞	∞
" succinate		3.0	5.4	∞	∞	∞	∞	∞	∞	∞
" citrate		4.2		∞	∞	∞	∞	∞	∞	∞
" acetate		2.7	20.0	∞	∞	∞	∞	∞	∞	∞
" monochloracetate		4.1	∞	∞	∞	∞	∞	∞	∞	∞
" dichloracetate		5.9	∞	∞	∞	∞	∞	∞	∞	∞
" trichloracetate	2.9			∞	∞	∞	∞	∞	∞	∞
" sulfate		6.8		9.0	11.2	12.8	14.6	14.4	13.2	13.3

by the addition of HCl, 5.5 cc. of alcohol were required for the same degree of opacity. When the pH of the 1 per cent gelatin chloride solution was only a trifle lower, namely 4.50, the addition of 25 cc. of alcohol or more did not suffice for bringing about the degree of opacity required for our test; only a lower degree of turbidity resulted. A gelatin chloride solution of pH 4.45 remained perfectly clear (with a bluish tint) regardless of how much alcohol was added. We may say that gelatin chloride becomes soluble in an alcohol-water mixture containing more than 75 per cent alcohol as soon as its pH is $\leqq$ 4.45.

It seemed of interest to compare the relative solubility of other gelatin-acid salts with that of gelatin chloride. Table IV gives the

result. The figures indicate the number of cc. of 95 per cent alcohol which when added to 10 cc. of 1 per cent gelatin solution brings about the standard degree of opacity. When the addition of 30 cc. or more alcohol to 10 cc. of the 1 per cent solution of gelatin-acid salt leaves the solution perfectly clear we indicate this by the sign ∞.

The result (which agrees with the results of a previous publication by the writer[5]) is unequivocal: all those gelatin-acid salts in which the anion in combination with gelatin is monovalent can no longer be precipitated by 95 per cent alcohol when the pH is $\leqq$ 4.4; while the only gelatin-acid salt in combination with a bivalent anion, namely gelatin sulfate, can be precipitated at any pH down to 2.0 (or even below). The relative solubility of gelatin-acid salts in alcohol shows,

TABLE V.

Cc. of 95 per cent alcohol required to bring 10 cc. of 1 per cent gelatin-salt solution to standard opacity.

	pH of metal gelatinate.							
	4.9	5.0	5.4	6.4	9.6	10.2	11.4	12.0
	cc.	cc.	cc.	cc	cc.	cc.	cc.	cc.
Li gelatinate	2.3	∞	∞	∞	∞	∞	∞	∞
Na "	2.0		∞	∞	∞	∞	∞	∞
K "	1.9	∞	∞	∞	∞	∞	∞	∞
NH_4 "	1.9	∞	∞	∞	∞	∞	∞	∞
Ca "		2.8	4.4	8.2	8.2	10.5	12.0	7.2
Ba "		2.1	4.2	6.6	7.9	8.1	5.9	5.3

therefore, the same influence of the valency (and lack of influence of the nature of the anion) which we have found in connection with the other properties of proteins like swelling, osmotic pressure, and viscosity.

The same agreement exists in regard to metal gelatinates. 10 cc. of a 1 per cent solution of Li, Na, K, and NH_4 gelatinate can no longer be precipitated by the addition of 95 per cent alcohol when the pH is $\leqq$ 5.0. The deviation from the isoelectric point is minute. 10 cc. of 1 per cent Ba and Ca gelatinate, however, can be precipitated with comparatively small quantities of 95 per cent alcohol at any pH (Table V).

[5] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 489.

We should expect that when the hydrogen ion concentration of a gelatin chloride solution becomes very high its solubility in an alcohol-water mixture will be diminished again. This is indeed the case, and happens when in 100 cc. of 2 per cent solution of isoelectric gelatin are contained 30 or 40 cc. of M/1 HCl. When to 5 cc. of such a solution are added 25 or 20 cc. of 95 per cent alcohol, the turbidity occurs again. When 100 cc. of the solution contain 50 cc. of M/1 HCl only 14.7 cc. of 95 per cent alcohol are required.

The same result was obtained with Na gelatinate which can also be precipitated again by alcohol when its pH exceeds 12 or 13.

The fact that the gelatin-acid salts (with the exception of gelatin sulfate) become completely soluble in alcohol when the pH reaches the low value of 4.4 is not easy to harmonize with the hypothesis of Pauli that this is due to the ionization of the gelatin, since the relative amount of ionized gelatin is exceedingly small at pH 4.4.

The experiments on the relative solubility of different gelatin salts therefore show the same influence of the valency of the ion in combination with gelatin as was shown in regard to the other physical properties of proteins.

III. Conductivity and Ionization of Gelatin Solutions.

The influence of ions on the conductivity of protein solutions should run parallel to the influence on swelling, viscosity, and osmotic pressure, if it be true that these properties depend on the concentration of the protein ions in the solution. According to this theory, first proposed by Laqueur and Sackur[6] and elaborated by Pauli,[7] the values for the physical properties of proteins are a minimum at the isoelectric point for the reason that the ionization of the protein molecules is a minimum at that point. When we add acid, *e.g.* HCl, protein chloride is formed which is highly ionized and the increase in the viscosity, swelling, and osmotic pressure with the increase of acid is explained by the ionization theory on the assumption of an increase in the concentration of the protein ions in the solution. When, however, too much acid is added, *i.e.* as soon as the pH of the gelatin solution

[6] Laqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[7] Pauli, W., Kolloidchemie der Eiweisskörper, pt. 1, Dresden and Leipsic, 1920

falls below 3.3, the swelling, osmotic pressure, and viscosity of the solution diminish again upon the addition of further acid. This would be explained by the ionization theory on the assumption that the concentration of ionized protein in the solution reaches a maximum at a pH of about 3.3, and that a further increase of acid lowers the concentration of ionized gelatin in the solution. The same theory should also explain the fact that the curves for the physical properties of gelatin salts with a bivalent ion are so much lower than the gelatin salts with a monovalent ion by the assumption that the latter are more highly ionized than the former.

We can determine the concentration of ionized gelatin in solution with the aid of conductivity measurements of the solution of a gelatin salt, *e.g.* gelatin chloride, if we deduct the conductivity of the free HCl in the solution from the total conductivity of the gelatin solution, since our gelatin solutions contain no other electrolyte except the free acid, *e.g.* HCl, and the gelatin salt; *e.g.* gelatin chloride. This is proved by the fact that at the isoelectric point our gelatin solutions had practically the conductivity zero (Figs. 4, 6, 8, and 9). Our method of procedure was as follows: doses of 1 gm. of powdered gelatin were brought to the isoelectric point and to each gram of isoelectric gelatin were added different quantities of 0.1 N acid or alkali and some water; the mass was melted by heating to 40° and then so much H_2O was added that the volume of the solution was 100 cc. After that the pH of the gelatin solution and the conductivities were determined.

Fig. 4 gives the curves for such measurements in the case of gelatin chloride. The abscissæ are the pH, the ordinates the specific conductivities multiplied by 10^4. The curve to the right is the total specific conductivity $\times 10^4$ of the gelatin chloride solution of different pH. The curve to the left represents the measurements of the specific conductivities $\times 10^4$ of pure HCl solutions (without gelatin) for different pH. By deducting the ordinates of this latter curve from the ordinates of the curve for total conductivity we get the curve in the middle representing the specific conductivity $\times 10^4$ of the pure gelatin chloride solution. Since it had been shown before that the viscosity of the solution does not influence the conductivity in this case (Hardy,

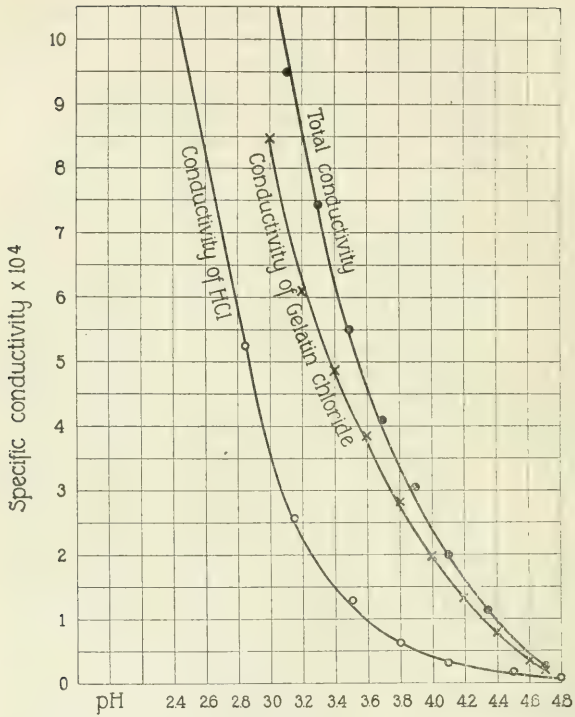

FIG. 4.

FIGS. 4 and 5. Specific conductivity of gelatin chloride solutions of different pH (but all 1 per cent in regard to isoelectric gelatin). Abscissæ are the pH, ordinates specific conductivity $\times 10^4$. Total conductivity means specific conductivity $\times 10^4$ of the gelatin solution measured directly. From this is to be deducted the specific conductivity of HCl of the same pH as the gelatin solution, to obtain the real curve for the specific conductivity of gelatin chloride.

Loeb, Northrop[8]), we may conclude that the middle curve represents the specific conductivity of the gelatin chloride solution and that it

[8] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 605.

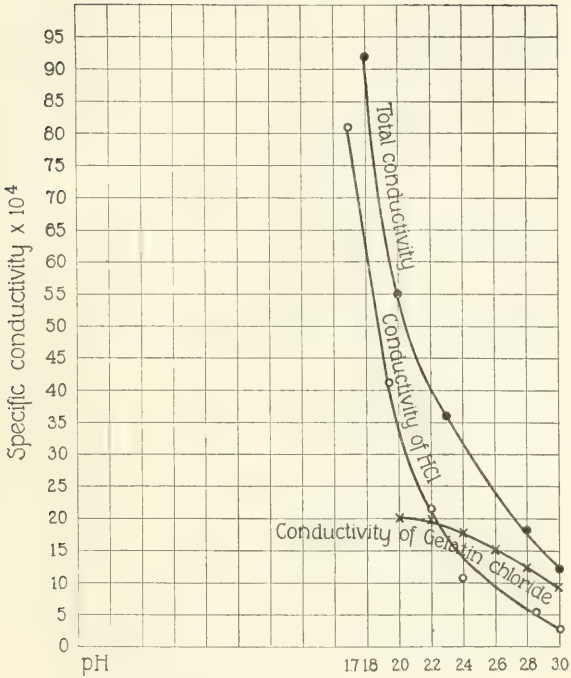

FIG. 5.

can hence be used as a measure of the concentration of the ionized gelatin in the solution. Fig. 4 shows that the curve for the conductivity of gelatin chloride rises continually with increasing hydrogen ion concentration. Fig. 5 is a completion of Fig. 4 for pH down to 2.0. (The ordinates are on a smaller scale in Fig. 5 than in Fig. 4.) It is obvious that at no time does the conductivity curve for gelatin chloride, *i.e.* the curve representing the concentration of ionized protein, show the drop observed in the curves representing the other properties of proteins.

Figs. 6 and 7 show that the same is true for the conductivity curve for gelatin sulfate; Fig. 6 gives the specific conductivities for pH 4.7 to 3.0, and Fig. 7 for pH 3.0 to 2.2 (the ordinates in Fig. 7 are on a

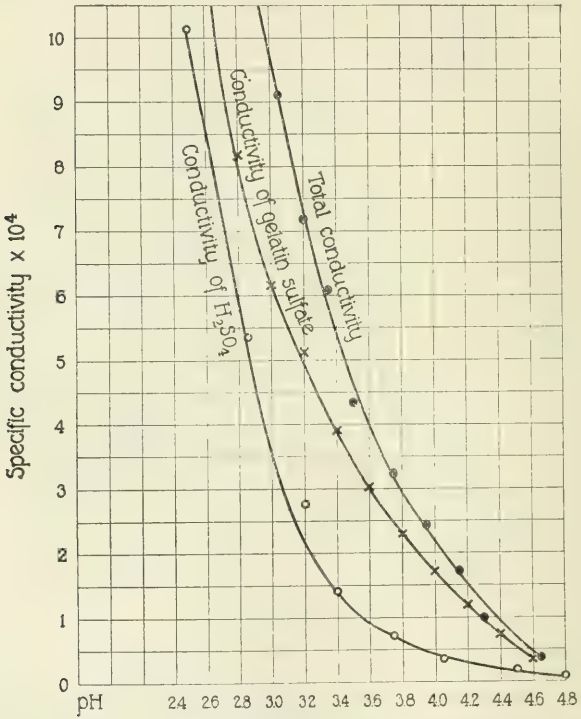

FIG. 6.

FIGS. 6 and 7. Conductivity curves for gelatin sulfate. See legend for Figs. 4 and 5.

smaller scale than in Fig. 6). Experiments on the conductivity of gelatin acetate, trichloracetate, phosphate, and oxalate all give a

similar result. These experiments do not support the hypothesis that the drop in the curves for viscosity, swelling, and osmotic pressure of gelatin-acid salts at or near pH 3.3 is due to a corresponding drop in the degree of ionization of the gelatin salts mentioned.

No drop was discovered in the conductivity curves for metal gelatinates (Na gelatinate, Fig. 8, and Ba gelatinate, Fig. 9).

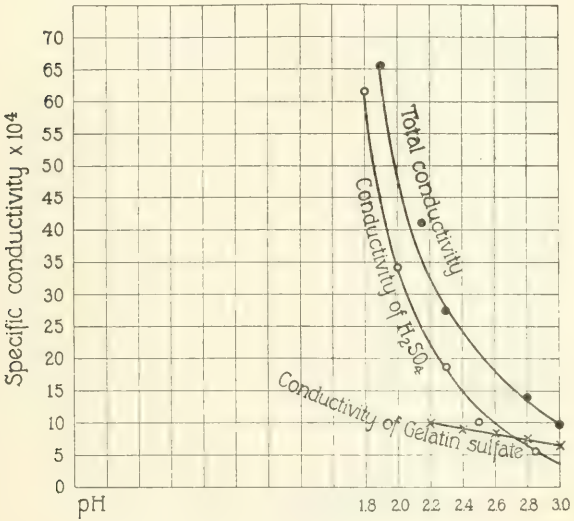

FIG. 7.

The question now arises whether we can explain the difference in the swelling, osmotic pressure, and viscosity of gelatin sulfate on the one hand and gelatin chloride and oxalate, etc., on the other hand on the basis of the ionization theory. If the ionization theory is correct the conductivity of gelatin oxalate, and of gelatin chloride should be twice or almost two and one-half times as great as that of gelatin sulfate. Yet Table VI shows that there is very little difference

between the conductivities of gelatin oxalate and gelatin sulfate; and also a difference of only 20 per cent between gelatin sulfate and gelatin chloride at pH 3.7. As a matter of fact the difference in conduc-

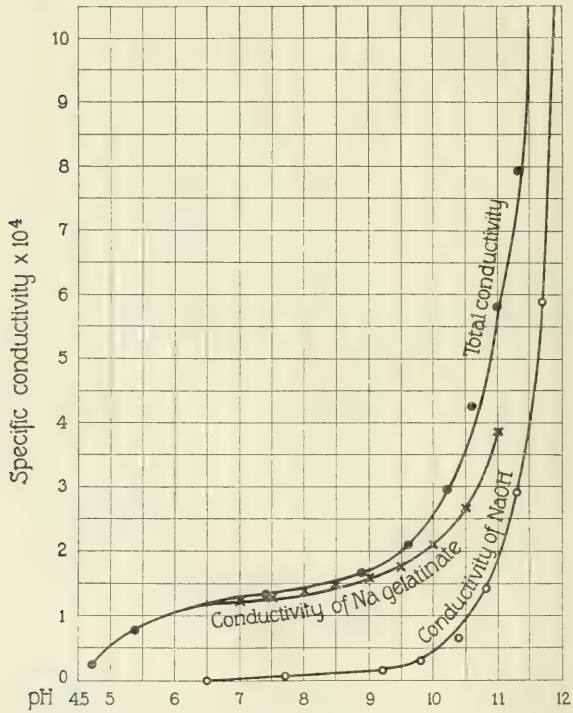

FIG. 8. Conductivity curve for Na gelatinate.

tivity between gelatin oxalate and gelatin chloride which show equal swelling, viscosity, and osmotic pressure is greater than the difference in conductivity between gelatin chloride and gelatin sulfate which are so enormously different in regard to swelling, osmotic pressure, etc.

The three salts, gelatin chloride, sulfate, and oxalate were chosen, since the ionic mobilities of Cl, $\frac{1}{2}$ SO_4, and $\frac{1}{2}$ oxalate are so nearly alike.

It had been pointed out by the writer in a previous paper that the difference in conductivities of Na and Ba gelatinate and of gelatin

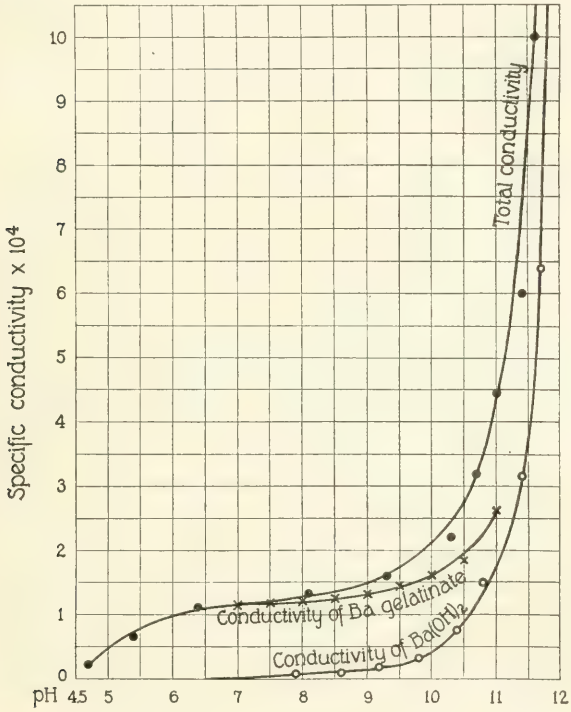

FIG. 9. Conductivity curve for Ba gelatinate.

bromide and gelatin sulfate is too small to account for the difference in the osmotic pressure of solutions of these two types of gelatin salts on the basis of differences in the ionization of the two protein salts[9]

[9] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 483, 569.

These data lend no support to the assumption that the difference between the swelling, viscosity, and osmotic pressure of gelatin sulfate on the one hand, and of gelatin chloride and gelatin oxalate on the other is due to differences in the degree of ionization of proteins.

TABLE VI.

Specific Conductivity of 1 per cent Solutions of Gelatin Chloride, Gelatin Sulfate, and Gelatin Oxalate.

	pH of gelatin-acid salt.				
	4.4	4.0	3.7	3.4	3.0
Gelatin oxalate	0.65	1.45	2.15	3.15	5.25
" sulfate	0.75	1.75	2.60	3.95	6.15
" chloride	0.8	2.0	3.25	4.85	8.5

SUMMARY AND CONCLUSIONS.

1. Our results show clearly that the Hofmeister series is not the correct expression of the relative effect of ions on the swelling of gelatin, and that it is not true that chlorides, bromides, and nitrates have "hydrating," and acetates, tartrates, citrates, and phosphates "dehydrating," effects. If the pH of the gelatin is taken into consideration, it is found that for the same pH the effect on swelling is the same for gelatin chloride, nitrate, trichloracetate, tartrate, succinate, oxalate, citrate, and phosphate, while the swelling is considerably less for gelatin sulfate. This is exactly what we should expect on the basis of the combining ratios of the corresponding acids with gelatin since the weak dibasic and tribasic acids combine with gelatin in molecular proportions while the strong dibasic acid H_2SO_4 combines with gelatin in equivalent proportions. In the case of the weak dibasic acids the anion in combination with gelatin is therefore monovalent and in the case of the strong H_2SO_4 it is bivalent. Hence it is only the valency and not the nature of the ion in combination with gelatin which affects the degree of swelling.

2. This is corroborated in the experiments with alkalies which show that LiOH, NaOH, KOH, and NH_4OH cause the same degree of swelling at the same pH of the gelatin solution and that this swell-

ing is considerably higher than that caused by $Ca(OH)_2$ and $Ba(OH)_2$ for the same pH. This agrees with the results of the titration experiments which prove that $Ca(OH)_2$ and $Ba(OH)_2$ combine with gelatin in equivalent proportions and that hence the cation in combination with the gelatin salt with these two latter bases is bivalent.

3. The fact that proteins combine with acids and alkalies on the basis of the forces of primary valency is therefore not only in full agreement with the influence of ions on the physical properties of proteins but allows us to predict this influence qualitatively and quantitatively.

4. What has been stated in regard to the influence of ions on the swelling of the different gelatin salts is also true in regard to the influence of ions on the relative solubility of gelatin in alcohol-water mixtures.

5. Conductivity measurements of solutions of gelatin salts do not support the theory that the drop in the curves for swelling, osmotic pressure, or viscosity, which occurs at a pH 3.3 or a little less, is due to a drop in the concentration of ionized protein in the solution; nor do they suggest that the difference between the physical properties of gelatin sulfate and gelatin chloride is due to differences in the degree of ionization of these two salts.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

I. Proteolytic Enzymes.

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Study of the biology of pneumococcus has led to a knowledge of certain biochemical characters which are common to the species as a whole, and to the recognition of fixed antigenic properties which serve to distinguish type differences within the species. The antigenic properties are inherent in the specificity of the bacterial protein and are detectable only by serologic reactions by means of which type relationships are recognized. The biochemical characters, on the other hand, are possessed in common by most pneumococci regardless of type differences, and are intimately associated with the life processes of the organism. These metabolic functions, upon which life of the cell depends, are in most instances referable to enzyme action. The presence or absence of a particular enzyme or group of enzymes determines largely the cellular activities of a microorganism. With the hope, therefore, of acquiring a better understanding of the way in which pneumococci adapt themselves to different environments, both in satisfying their nutritional needs and in exhibiting their invasive properties, the present study on the nature of the intracellular enzymes of this organism has been undertaken.

In the isolation and study of the enzymes of pneumococcus, apart from the living cell to which they are so intimately bound, use has been made of the fact that this organism undergoes rapid and complete solution in the presence of bile. Moreover, bile dissolves the bacterial cell with little or no change in the specific antigenic substance and with little or no injury to other demonstrable intracellular substances, such as the endohemotoxin. By dissolving the pneumococcus in bile and testing the cell-free solution on suitable substrates, enzymes are readily demonstrable. These enzymes have been found

to possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids. In demonstrating carbohydrate cleavage, however, bile was found to inhibit completely the hydrolysis of sucrose and starch, and another method of preparing the enzyme solution was necessary. This point will be emphasized in the paper on the intracellular invertase and amylase of pneumococcus (1).

The present paper concerns itself with the study of the proteolytic enzymes of pneumococcus. The intracellular nature of the enzymes, the influence of hydrogen ion concentration, the effect of age and concentration of the enzyme upon activity, and the relation of these enzymes to the virulence of the organism and to the mechanism of bile solubility will be discussed.

EXPERIMENTAL.

Bacteriological Methods.

Media.—The beef infusion broth containing 1 per cent peptone was prepared as previously described (2) except that (*a*) 2 gm. of dibasic phosphate per liter—anhydrous sodium phosphate or potassium phosphate—were used instead of 5 gm. per liter of sodium chloride, and (*b*) the medium was adjusted to a pH of 7.8.

Bile.—The ox bile used in preparing the pneumococcus enzyme solution was autoclaved, filtered, and again autoclaved as previously described (2).

Sterility Controls.—No antiseptics were used. The sterility of the enzyme solution was tested in broth and on blood agar plates. After addition of enzyme solution to the substrate, cultures of the mixtures were made by adding 0.1 cc. to 5 cc. of plain broth. In all the experiments recorded these controls remained sterile.

Chemical Methods.

Preparation of Substrate Solutions.—2 per cent solutions of peptone or protein were made in distilled water and the reaction was adjusted to the desired pH. The 2 per cent solution was then diluted with

an equal volume of 0.1 M phosphate solution of the desired pH. The final concentrations, unless otherwise stated, were therefore 1 per cent of substance in 0.05 M phosphate solution. The phosphate solutions were prepared from Merck's special reagents (Na_2HPO_4 and KH_2PO_4) according to Sörensen's (3) tables.

Sterilization.—Unless otherwise stated, sterilization was accomplished by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The pH values were usually determined colorimetrically, with the series of indicators outlined by Clark and Lubs (4). The solutions were diluted with two volumes of redistilled water, and the indicators used in such strength that one drop was required per 3 cc. of diluted solution. The readings were made by the comparator method (Walpole). Sörensen's standard phosphate and Walpole's (5) standard acetate solutions were used. These determinations were frequently checked by the electrometric method.

Nitrogen Determinations.—Total nitrogen determinations were made by the Kjeldahl method. Amino nitrogen was determined by Van Slyke's (6) nitrous acid method. With the peptone solution the determinations were made directly on 2 cc. samples, by means of the micro apparatus. Determinations of the amino nitrogen of the protein solutions were done by one of two methods: (*a*) 10 cc. samples were deaminized for 15 minutes in the large Van Slyke apparatus, and the nitrogen liberated was read in the micro burette calibrated to 0.002 cc.; and (*b*) the protein was precipitated with colloidal iron in the manner described by Van Slyke, Vinograd-Villchur, and Losee (7).

In determining the peptide nitrogen of the peptone solutions the peptides were split to amino-acids by acid hydrolysis (Van Slyke (8)). The peptide nitrogen was then calculated as the increase in amino nitrogen.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Fairchild).

Experiment 1. (*a*) *Preparation of Enzyme.*—The washed bacterial residue from 2 liters of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was taken up in 15 cc. of 33 per cent dilution of sterile bile (bile, 5 cc., + water, 10 cc.) and placed in the ice box over night. A portion of this bile solution of pneumococcus was inactivated by heat, as a control.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution of various hydrogen ion concentrations were sterilized by the Arnold method on 3 successive days.

(*c*) *Sterility Control.*—No antiseptics were used. After the addition of enzyme solution to substrate, cultures of all digestive mixtures were made by adding 0.1 cc. of each to 5 cc. of plain broth. All cultures, including that of the bile solution of pneumococcus, were sterile.

The experiment was carried out as follows: Duplicate 20 cc. portions of the peptone substrate at reactions of pH 4.2, 5.3, 6.2, 7.0, and 7.6 were prepared. To one tube of each set, 1 cc. of enzyme solution was added, to the other 1 cc. of the inactivated enzyme solution. The tubes were then kept at 37°C. for 2 days. No antiseptic was used, sterility being maintained throughout by bacteriological technique. All cultural controls remained sterile, and after 48 hours the tubes were removed from the incubator for analysis. Duplicate amino-acid nitrogen determinations were made on 2 cc. samples by the Van Slyke method. The estimations of hydrogen ion concentration were determined colorimetrically on 5 cc. portions. The results are tabulated in Table I.

TABLE I.

Determination of Peptone-Splitting Activity (Fairchild's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.2	4.2	27.3	27.2	
2	5.3	5.3	27.5	32.3	4.7
3	6.2	6.2	27.6	45.3	17.7
4	7.0	7.1	29.5	49.8	20.3
5	7.6	7.6	28.2	47.9	19.6
Peptone.			28.2		

Distribution of Nitrogen per 100 Cc. of Substrate.

		mg.
Total nitrogen		107
Amino "	before hydrolysis	27.5
" "	after "	78.6
Peptide "	therefore equals	51.1

Analysis of Experiment.

The increase of 20.3 mg. of amino nitrogen per 100 cc. of substrate shows that 40 per cent of peptide nitrogen was split to free amino nitrogen.

In order to determine the proportion of the peptide that was digested, a nitrogen partition was done on the peptone solution. Total nitrogen on 2 cc. portions was determined by the Kjeldahl method. Peptide nitrogen[1] was computed as the difference between total amino-acid nitrogen before and after hydrolysis.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Witte).

Experiment 2. (*a*) *Preparation of Enzyme.*—This was the same as in Experiment 1, except that the bacterial residue from 2 liters of Pneumococcus Type II (No. F 208) was dissolved in 15 cc. of 25 per cent solution of sterile bile in water.

(*b*) *Preparation of Substrate.*—The substrate was prepared as in Experiment 1, except that Witte's peptone was used instead of Fairchild's preparation.

(*c*) *Sterility Control.*—No antiseptic was used. All tubes including cultural control of the enzyme solution were proved sterile by subcultures as in the preceding experiment.

In Experiment 2 the peptone-splitting action of pneumococcus enzyme was tested on Witte's peptone. The experimental technique was the same as that described in the preceding protocol, except for the substitution of 1 per cent Witte's peptone for the Fairchild preparation. The Witte peptone substrate was analyzed for total nitrogen, and peptide nitrogen in the same manner. The results are given in Table II.

It is evident from Experiments 1 and 2 that pneumococcus contains within its cell an enzyme or enzymes capable of hydrolyzing peptides into amino-acids or simpler peptides. From 26 to 40 per cent of the peptide nitrogen present in the peptone substrate was split to amino nitrogen. The term "peptone" solution is used to indicate the mixtures of partially hydrolyzed protein products which are known commercially as "peptones." The considerable data available as to the chemical nature of these peptones show them to be mixtures of protein products of varying degrees of complexity. The amount of pep-

[1] By peptide nitrogen is meant nitrogen found in the peptide linkings, the $-CO-NH-$ groups that link the different amino-acids together in peptides, proteins, or intermediate products. The process of hydrolysis consists in the splitting of these peptide groups, from each of which is generated a carboxyl group and an amino group. Thus

$$RNH-CO-R^1 + H_2O = RNH_2 + R^1COOH$$

For further discussion of this point see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

tide hydrolyzed was greater in the experiment in which Fairchild's peptone was used. The proportion of preformed amino nitrogen to total nitrogen in this preparation was greater than in the sample of Witte's peptone, indicating that, on the average, Fairchild's peptone consists of simpler intermediate protein digestion products than Witte's. This may be the reason that further digestion by the enzyme proceeded more rapidly in the Fairchild product, the enzyme attacking the simpler peptides of the preparation the more readily.

TABLE II.

Determination of Peptone-Splitting Activity (Witte's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.4	4.4	15.5	15.6	
2	5.0	5.0	15.6	21.2	5.6
3	6.0	6.0	15.4	32.4	17.0
4	7.0	7.0	15.5	39.6	24.1
5	7.8	7.8	15.5	39.4	23.9

Distribution of Nitrogen per 100 Cc. of Substrate.

		mg.
Total nitrogen		148
Amino "	before hydrolysis	15.5
" "	after "	108
Peptide "		92.5

Analysis of Experiment.

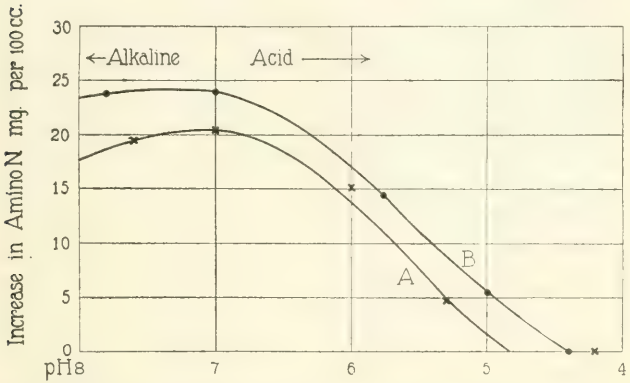
The increase of 24.1 mg. per 100 cc. of substrate shows that 26 per cent of peptide nitrogen was split to free amino nitrogen.

Effect of Hydrogen Ion Concentration on the Activity of Pneumococcus Peptonase.

Experiments 1 and 2 were planned to determine the relation of enzyme activity to hydrogen ion concentration. This relation is evident from the curves of Text-fig. 1, in which *A* represents Experiment 1 and *B* Experiment 2. The optimum activity of the peptone-splitting enzyme is from pH 7 to pH 7.8. With increase in acidity the

activity of the enzyme is increasingly retarded until complete inhibition results at pH 4.5.

These facts, that about 30 to 40 per cent of the peptide is hydrolyzed and that the optimum zone for activity of the enzyme is between pH 7 and 7.8, place it in the erepsin class of enzymes. Moreover, the fact that the curve of acid inhibition proceeds in a straight line to complete inhibition at a pH of about 4.5 indicates that the enzyme preparation used contains no pepsin and is, therefore, not a complex



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of pneumococcus peptonase. Curve *A*, Experiment 1; Curve *B*, Experiment 2.

of erepsin- and pepsin-like enzymes. Since it seemed desirable to maintain an open mind on the question as to whether the enzymotic action in these experiments is more closely allied to trypsin or erepsin, and in view of its relative rate of action on native protein, the term "peptonase" has been used throughout the remainder of this report. It is significant that the optimum reaction zone for the intracellular peptolytic enzyme corresponds with the optimum for growth of pneumococcus (9).

Use of Sodium Choleate in Demonstrating the Intracellular Enzymes of Pneumococcus.

It is well known that pneumococci undergo solution in the presence of bile salts as completely as in the presence of bile itself. In order to determine whether the substitution of sodium choleate for bile in dissolving the organisms exerted any influence on the activity of the intracellular enzymes the following experiment was carried out.

Experiment 3. (*a*) *Preparation of Enzyme.*—Pneumococcus Type I (No. G_2) was grown in 2 liters of plain broth for 18 hours at 37°C. The bacteria were removed by centrifugation, washed once in sterile isotonic salt solution, and suspended in 10 cc. of 5 per cent solution of sodium choleate. After 5 hours in a water bath at 37°C., the resultant solution of pneumococci was diluted with an equal volume of sterile distilled water. One portion of this solution was inactivated by heat and both were tested for activity in a peptone substrate.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution were adjusted to the various hydrogen ion concentrations and sterilized by the Arnold method on 3 successive days.

This experiment was conducted in exactly the same manner as Experiment 1.

TABLE III.

Determination of Peptone-Splitting Activity of a Solution of Pneumococcus Obtained by the Use of Sodium Choleate.

Tube No.	pH	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
		mg.	*mg.*	*mg.*
1	5.0	37.9	42.9	5.0
2	5.4	37.9	50.7	12.8
3	5.8	38.7	53.4	14.7
4	6.2	40.4	55.2	14.8
5	6.6	41.6	59.2	17.6
6	7.0	42.2	60.3	18.1
7	7.4	39.6	58.9	19.3
8	7.8	40.7	58.3	17.6

From the data presented in Table III it is evident that when pneumococci are dissolved by sodium choleate there is liberated from the cell a peptone-splitting enzyme in the same manner as when solution of the organism is effected by the action of bile. It has also been

found that enzyme solutions obtained by disintegration of pneumococcus cells without the presence of bile or bile salts, by methods described in a succeeding paper (1), exhibit comparable activity. Therefore, bile salts are not essential to the action of the enzyme.

Effect of Age on the Activity of the Intracellular Peptonase of Pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme.*—The technique of preparing the enzyme solution in this experiment was similar to that described in preceding protocols, except that the bacterial residue from 2 liters of broth culture of Pneumococcus Type II (No. F 208) was dissolved directly in 10 cc. of undiluted bile. After 2 hours in the water bath at 37°C. and 1 hour at room temperature, the enzyme solution was stored in the ice box, and at intervals up to 43 days portions were removed and tested for activity.

(*b*) *Preparation of Substrate.*—1 per cent of Fairchild's peptone in $\frac{M}{30}$ phosphate solution of pH 7 was prepared and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

(*c*) *Sterility Control.*—No antiseptics were used. Cultures of the original enzyme solution and each digestion mixture at the time of carrying out the several tests proved sterile.

The results are presented in Table IV.

TABLE IV.

Age Stability of Endopeptonase.

Enzyme solution kept at about 4°C. 1 cc. added to 20 cc. of peptone (Fairchild), pH 7. 24 hours at 37°C.

Age.	Amino nitrogen per 100 cc of substrate		
	Inactive.	Active.	Increase.
days	*mg.*	*mg.*	*mg.*
0	37.4	58.8	21.4
1	37.4	59.7	22.3
2	40.8	62.6	21.8
6	37.4	53.6	16.2
20	40.8	54.4	13.6
43	23.4	32.1	8.7

The length of time an enzyme may remain active is dependent upon the conditions of its preservation. The age stability of an

enzyme in dried form is greater than that of the same enzyme in solution. In the present instance, the intracellular peptonase of pneumococcus dissolved in undiluted ox bile retained about 40 per cent of its activity for over 6 weeks.

Relation of Virulence of Pneumococcus to Enzyme Activity.

Experiment 5. (*a*) *Preparation of Enzyme.*—Plain broth cultures (1,500 cc.) of a virulent and avirulent strain of Pneumococcus Type II (No. F 208) were centrifuged; the bacterial sediment was washed once in sterile isotonic salt solution, then dissolved in 15 cc. of undiluted bile, and held in the ice box over night. Portions of the enzyme solutions were inactivated by heating in the autoclave at 15 pounds pressure for 20 minutes.

TABLE V.

Influence of Virulence of Pneumococcus upon the Activity of the Intracellular Peptonase.

2 cc. of enzyme solution added to 20 cc. of peptone solution, pH 7. Incubated at 37°C. for 24 hours.

Pneumococcus Type II.	Minimum fatal dose.	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
	cc.	*mg.*	*mg.*	*mg.*
No. F 208 A	0.000001	39.8	68.6	28.8
" F 208 "B"	Greater than 1	39.2	67.8	28.6

(*b*) *Preparation of Substrate.*—1 per cent peptone (Fairchild) in $\frac{M}{50}$ phosphate solution, pH 7, was sterilized in the autoclave.

(*c*) *Sterility Control.*—No antiseptics were used. Each tube in the experiment was tested for sterility by subculture and yielded no growth.

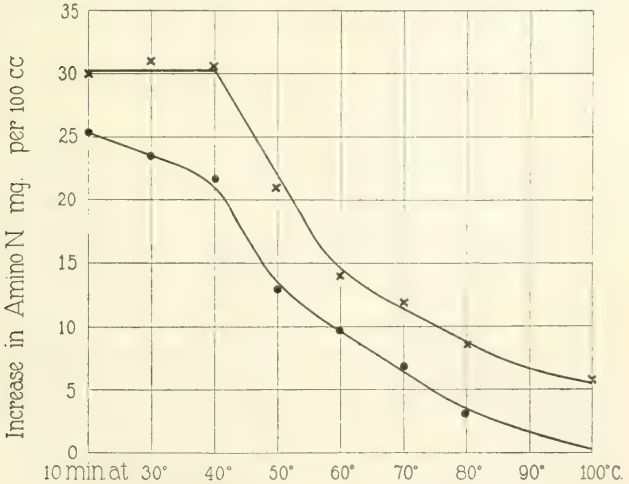
The development of a technique for the demonstration of endoenzymes made it possible to submit to experimental proof the question whether differences in virulence are in any way related to the activity of the intracellular enzymes. For this purpose a strain of Pneumococcus Type II (No. F 208) was chosen. This organism was originally isolated from the blood of a patient suffering from lobar pneumonia. The virulence of the strain, maintained by animal passage, was such that 0.000001 cc. of broth culture injected intraperitoneally into white mice proved fatal in 24 to 48 hours. A subculture of the same strain, the virulence of which had been attenuated by cultural methods, failed to kill mice in doses of 1 cc. Enzyme solutions from comparable amounts of bacteria were prepared from the virulent and avirulent cultures of this strain. The respective

enzyme preparations were tested for peptonase action by adding 2 cc. of each to substrates of 20 cc. of 1 per cent peptone solution adjusted by phosphates to pH 7. After 24 hours at 37°C. the degree of enzyme action was determined by measuring the increase of amino nitrogen as indicated in Table V.

From Table V it appears that loss of virulence is not associated with a corresponding loss of enzymotic activity. Under the conditions of this experiment at least, the amount of hydrolysis of peptone by the endoenzymes of the avirulent strain was equivalent to that of the virulent organism.

Effect of Heat on the Intracellular Peptonase of Pneumococcus.

Sensitiveness to heat is a biologic character of all enzymes. In determining the influence of heat upon dissolved enzymes, the degree of temperature, the length of exposure, and the reaction of the solu-



TEXT-FIG. 2. Heat stability of intracellular peptonase of pneumococcus. The lower curve represents the results after 24 hours incubation at 37°C., the upper curve after 48 hours at 37°C.

tion are closely interrelated. The optimum reaction for activity of the endopeptonase of pneumococcus, pH 7.4, and an exposure of 10 minutes were arbitrarily chosen, and the temperature alone was varied as shown in Text-fig. 2.

Experiment 6. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of plain broth culture of Pneumococcus Type II (No. F 208) was dissolved in 20 cc. of sterile ox bile and held in the ice box over night.

(*b*) *Preparation of Substrate.*—1 per cent peptone solution (Fairchild) in 0.05 M phosphate solution adjusted to pH 7.4 was sterilized in the autoclave.

(*c*) *Sterility Control.*—Sterility was proved by subculture from each tube.

1 cc. of the enzyme solution with a pH of about 7.4 was placed in each of eight sterile tubes, and these in turn were immersed in water baths at 30°, 40°, 50°, 60°, 70°, 80°, 90°, and 100°C., respectively, for exactly 10 minutes. On removal the tubes were immediately cooled, and to each were added 10 cc. of the sterile substrate. After 24 and 48 hours incubation at 37°C. samples were removed for analysis. The results are plotted in Text-fig. 2. The heat sensitiveness of the enzyme manifests itself in a progressive loss of activity after exposure for 10 minutes to increasing temperatures, until at 100°C. little or no activity remains.

Effect of Concentration of Enzyme on the Activity of the Intracellular Peptonase of Pneumococcus.

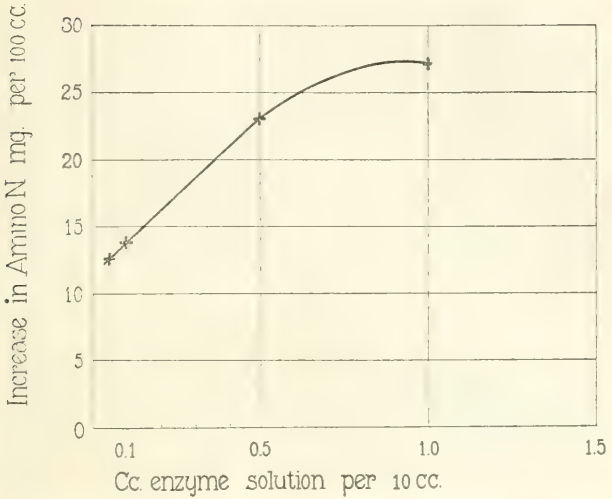
Experiment 7.—The same preparation of enzyme and substrate used in Experiment 6 was employed in this test, a bile solution of Pneumococcus Type II and a 1 per cent solution of peptone (Fairchild's) in 0.05 M phosphate mixture, pH 7.4.

The dissolved enzyme was diluted with bile so that 1 cc. of the final solution added to 10 cc. of peptone substrate contained 0.05, 0.1, 0.5, and 1 cc. respectively of the original enzyme solution. The results are plotted in Text-fig. 3.

For a more exact study of the dynamics of this enzyme it would be desirable to repeat this experiment with shorter digestion periods and smaller amounts of enzyme. It is evident, however, that under given conditions the rate of hydrolysis is proportional to the concentration of enzyme.

Occurrence of Enzymes in Culture Filtrates of Pneumococcus.

The experiments thus far have dealt entirely with intracellular enzymes. It was considered probable that the culture medium itself



TEXT-FIG. 3. Effect of concentration of enzyme on the activity of the intracellular peptonase of pneumococcus.

would contain similar enzymes which had either diffused out from the cell during growth or had been liberated by disintegration of the organisms in the culture fluid. In order to determine the validity of this assumption, the following experiment was undertaken.

Experiment 8. (*a*) *Preparation of Filtrate.*—100 cc. of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was filtered through a Berkefeld candle N. The filtrate, pH 7, was tested for sterility by adding 5 cc. to 100 cc. of fresh broth and incubating at 37°C. In testing the sterility of culture filtrates of pneumococcus it is not sufficient merely to incubate the filtrate itself, but it is essential to inoculate fresh broth. This is probably due to the fact, recorded in a previous paper (Avery and Cullen (10)), that further growth cannot be initiated in the filtrates of a plain broth culture of pneumococcus even though the reaction is readjusted to the optimum hydrogen ion concentration. A portion of the enzyme-containing filtrate was inactivated by heat, to serve as a control.

(*b*) *Preparation of Substrates.*—These consisted of the sterile culture filtrate and a 1 per cent solution of peptone (Fairchild) in phosphate mixture adjusted

to pH 7, the same hydrogen ion concentration as the filtrate. The peptone solution was sterilized in the autoclave.

In determining the presence of enzyme in culture filtrates of pneumococcus the amount of cleavage was determined, (*a*) as the result of further action of the enzyme on the peptones present in the broth filtrates and (*b*) as the result of this action plus the action on additional peptones. This was accomplished by the procedure indicated in Table VI, three tubes of substrate being used, in all of which the final volume was 20 cc. The first tube contained 10 cc. of filtrate and 10 cc. of sterile water, the second, 10 cc. of filtrate and 10 cc. of 1 per cent peptone solution, and the third (the control), 10 cc. of 1 per cent peptone solution plus 10 cc. of sterile water. The determinations of amino nitrogen in the digestion mixtures before and after incubation are given in Table VI.

TABLE VI.

Presence of Peptonase in Culture Filtrate of Pneumococcus.

Tube No.	Sterile filtrate unheated; pH 7.	1 per cent peptone solution; pH 7.	Water.	Amino nitrogen per 100 cc. of final solution.		
				Before digestion.	After digestion.	Increase.
	cc.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*
1	10		10	54.9	73.4	18.5
2	10		10	54.7	73.0	18.3
3		10	10	24.0	24.0	
4		10	10	24.0	24.0	
5	10	10		78.9	106.8	27.9
6	10	10		79.0	106.8	27.8

Analysis of Table VI.

Increase in amino nitrogen in filtrate alone..... 18.4 mg. per 100 cc.
" " " " " " + peptone.... 27.9 " " 100 "
" " " " due to action of enzyme
on added peptone..... 9.4 " " 100 "

From Table VI it is clear that under the conditions of the experiment the bacteria-free filtrate contained an active enzyme which continued to hydrolyze the excess of available peptide in the medium and in addition attacked the added peptone.

The demonstration of the presence of an enzyme in culture filtrates of pneumococcus after 18 hours incubation does not necessarily imply that the enzyme is a true secretory product which is elaborated during

growth and given off into the medium in the manner of exotoxins. Under optimum cultural conditions pneumococcus reaches its maximum growth relatively early (Chesney (11)), after which involution and disintegration of the bacterial cells soon begin. During this later period, the intracellular hemolysin of pneumococcus, which cannot be detected free in the medium during the early phase of active growth, is also released from the disrupted cell and can be demonstrated in the culture fluid. If the peptonase is an endoenzyme, its absence in filtrates during the period of active growth should be demonstrable.

In order, therefore, to test for presence of enzyme in the culture fluid before cell death and disruption with consequent liberation of intracellular enzyme occurred, the following experiment was carried out.

200 cc. of plain broth, pH 7.8, were inoculated with 0.5 cc. of a 5 hour culture of Pneumococcus Type II (No. F 208). After 5 hours incubation marked growth was apparent and the acidity had increased to pH 7.5. The culture was then centrifuged and the supernatant fluid was filtered through a tested Berkefeld candle N. This filtrate was kept in the refrigerator until its sterility was proved by culture. The sterile filtrate was then tested for enzyme activity as in the preceding experiment.

It was found that during the phase of active growth the culture fluid freed from bacteria possessed no peptolytic activity. Moreover, in this same culture fluid the intracellular hemolysin, known to be liberated by cell disintegration, was likewise not demonstrable, but the soluble substance shown by Dochez and Avery (12) to be elaborated during the earliest phase of cell multiplication was detectable in considerable concentration. The occurrence, therefore, of peptolytic activity in autolyzing broth cultures and its absence in the culture fluid during the early phases of active growth make it evident that the peptonase is a true endoenzyme.

Effect of Exposure to Acid Reaction on the Intracellular Peptonase of Pneumococcus.

It has been observed that cultures of pneumococci grown in sugar-containing medium reach a final hydrogen ion concentration of about pH 5 to 5.2. At this point the organisms quickly succumb, the acidity

produced by their own metabolic processes being sufficient in itself to stop growth. Lord and Nye (13) have found that pneumococci will not live for more than a few hours in a solution the acidity of which is greater than pH 5.1. Experiments in this laboratory have shown that pneumococci subjected to an acidity of pH 4.5 to 5 for 2 hours are no longer soluble in bile. Even when these organisms are removed by centrifugation, washed, and resuspended in phosphate solution at a pH 7.8, they remain insoluble in bile. The question arose whether this reaction of pH 4.5 to 5, which corresponds to the acid death-point of the bacterial cells, is also fatal to the intracellular enzymes, and whether this fact is of any significance in the phenomenon of bile insolubility.

To determine whether acid treatment injured the endoenzymes of pneumococcus the following experiment was carried out.

Experiment 9.—1 cc. of the enzyme solution used in Experiment 6 was treated with 4 cc. of 0.1 M potassium acid phosphate solution of pH 4.5. The reaction of the resulting solution was pH 5. After 2 hours at 37°C. the solution was readjusted to pH 7.4 by the addition of 0.3 cc. of N sodium hydroxide (calculated and verified on a separate sample), and 5 cc. of a 2 per cent peptone solution of pH 7.4 were added. As a control on activity, the untreated active enzyme was added to the same peptone substrate in similar proportions. The results are given in Table VII.

TABLE VII.

Effect on Peptonase of Exposure to Acid Reaction.

Final concentration of enzyme was 1 cc. per 10 cc. of 1 per cent peptone in 0.1 M phosphate solution of pH 7.4. 42 hours at 37°C.

Treatment of enzyme.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
	mg.	*mg.*	*mg.*
Enzyme solution previously kept at pH 7.8.....	31.2	58	26.8
" " " adjusted to pH 5.0 and after 2 hrs. at 37°C. readjusted to pH 7.4.....	31.2	58	26.8

The intracellular enzyme in solution suffered no loss of potency after being subjected for 2 hours to an acidity of pH 5, for upon readjustment to the optimum hydrogen ion concentration of pH 7.4, the

acid-treated enzyme exhibited an activity comparable to that of the untreated enzyme. The endopeptonase of pneumococcus is evidently little influenced by this reaction change. The bile insolubility of pneumococci at the acid death-point is, therefore, not associated with destruction of this enzyme, but is probably referable to coagulative changes in the cell protoplasm.

Action of the Endoenzymes of Pneumococcus on the Proteins, Casein, Gelatin, Albumin, and Fibrin.

The data presented in the preceding protocols establish the fact that there is present within the bacterial cell an enzyme, or enzymes, capable of hydrolyzing peptones into amino-acids, and that this enzyme-complex manifests its optimum activity in a slightly alkaline medium. Simultaneous experiments were carried out with the same enzyme preparations to determine their action on the proteins, casein, gelatin, fibrin, and albumin.

Preparation of Enzyme.—In these experiments the enzyme solutions were prepared in the manner described in Experiment 6.

Preparation of Substrates.—2 per cent gelatin, albumin (egg), and casein solutions were adjusted to pH 7.4 and diluted with an equal volume of 0.1 M phosphate solution of the same hydrogen ion concentration and then sterilized in the autoclave. The fibrin substrate D was prepared by adding 0.2 gm. of dried commercial fibrin to 10 cc. of 0.05 M phosphate solution of pH 7.4 and was sterilized in the autoclave. The fibrin substrate F was freshly prepared from 10 cc. portions of sterile oxalated rabbit plasma. Sterile calcium chloride was added and the fibrin clot was washed in sterile salt solution and transferred to 10 cc. of sterile 0.05 M phosphate solution of pH 7.4.

Experiment 10.—To 10 cc. portions of the sterile protein solution 1 cc. of enzyme solution was added, and the mixture placed at 37°C. for 5 to 7 days. The degree of digestion was determined as in the peptone experiments by amino nitrogen determination with one of the following procedures: (*a*) 10 cc. portions of the digestion mixtures were placed in the deaminizing bulb of the original large type of Van Slyke apparatus. 15 minutes were allowed for deamination and the nitrogen evolved was measured in a micro burette graduated to 0.002 cc. (*b*) After removal of the proteins by colloidal iron, amino nitrogen determinations were made in the manner described in preceding experiments. The results are presented in Table VIII.

Experiment 11.—In following the extent of digestion of the peptone solution control amino nitrogen determinations on active enzyme alone had shown that

TABLE VIII.

Proteolytic Action of Endoenzymes of Pneumococcus.

Protein.	Duration of digestion.	Method of analysis.	Amino nitrogen per 100 cc. of substrate.		
			Inactive.	Active.	Increase
	days		*mg.*	*mg.*	*mg.*
Casein.	5	(*b*)	1.9	6.3	4.4
"	12	(*b*)	1.3	3.4	2.1
Gelatin.	7	(*a*)	6.3	6.7	0.4
	12	(*b*)	2.8	3.5	0.7
Fibrin D.	5	(*a*)	2.5	4.0	1.5
" F.	31	(*b*)	4.2	7.1	2.9

the increase in amino nitrogen due to autolysis of the bacterial proteins was negligible. However, because of the small concentration of free amino nitrogen in the protein experiment more rigid control of this protein digestion was carried out. Duplicate 10 cc. portions of casein, albumin, and 0.05 M phosphate, all at pH 7.4, were prepared. To one tube of each pair 2 cc. of active enzyme were added, and to the other 2 cc. of heated enzyme. Tubes were incubated at 37°C. for 5 days. Each dilution was then diluted to 25 cc., 5 cc. portions were removed for total nitrogen determinations, and the remaining 20 cc. were freed from protein by precipitation with colloidal iron. The non-protein nitrogen of the filtrate and washings was determined by the Kjeldahl method. The results are presented in Table IX.

TABLE IX.

Proteolytic Action of Endoenzymes of Pneumococcus.

Proteolysis measured by increase in non-protein nitrogen. Digestion for 5 days at 37°C.

Solution.	Total nitrogen per 100 cc. of solution.	Non-protein nitrogen per 100 cc. of solution.			Protein digested.
		Inactive.	Active.	Increase.	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Enzyme	25.7	14.8	16.8	2.0	20.0
Casein*	14.6	7.0	19.4	12.4	8.9
Albumin*	12.3	6.4	6.5		

* Protein figures corrected for nitrogen of enzyme solution added.

From Table IX it is evident that autolysis of the enzyme solution itself is occurring. This increase would, for the concentrations used in Table VIII, amount to about 0.5 mg. of amino nitrogen per 100 cc.; that is, the increase in the case of the gelatin experiment is due to the enzyme solution. The increase of 3 and 4 mg. in the case of casein and fibrin indicates a definite proteolysis.

The endoenzymes of pneumococcus are apparently able partially to hydrolyze the proteins, casein and fibrin, but not albumin or gelatin. This enzyme action on intact protein is distinctly less, however, than that which occurs in the presence of peptones.

DISCUSSION.

To present a critical review of the literature on the nature and action of bacterial enzymes would entail a task beyond the scope of this paper. Although extensive study of the enzymes of a large variety of different species of microorganisms has been made, comparatively little work has been done on the occurrence and character of the endoenzymes of pneumococcus.

Rosenow (14) demonstrated in extracts of virulent pneumococci and filtrates of broth cultures a proteolytic enzyme capable of hydrolyzing the proteins in meat broth, in ascites meat broth, and to a less extent the proteins of heated serum. He prepared extracts of pneumococci by suspending the bacteria from broth cultures in salt solution, adding ether, and allowing autolysis to proceed at 37°C. for 48 hours. The bacteria were removed by centrifugation or passage through a Berkefeld filter. Rosenow found that the degree of disintegration of the bacterial cells was directly proportional to the amount of proteolysis as measured by formol titration. He has shown further that the toxicity of broth, culture filtrates, and extracts of pneumococci is associated with proteolysis due to the dissolved enzymes.

Evidence is presented in this paper of the existence of proteolytic enzymes within the cell body of pneumococcus. This enzyme or group of enzymes can be isolated from the living cell by dissolving the organisms in bile or allowing them to cytolyze in phosphate solutions of pH 6.2. In the latter method alternate thawing and freezing of the bacterial suspension greatly facilitate the extraction process by disrupting the bacterial cells.[2] By these methods the intracellular

[2] See Avery and Cullen (1).

substances pass into solution in a medium in which they retain their activity for a considerable period. The proteolytic activity of these enzymes is manifest in their ability to hydrolyze, to some extent, intact protein and to split to a striking degree intermediate products, such as peptones, into simpler peptides and amino-acids. It has not been determined whether the two processes, proteolysis and peptolysis, are functions of the same enzyme or the result of the action of two separate enzymes.

It is evident that the action on the intact proteins, fibrin and casein, is distinctly less than on simpler substances such as occurs in peptone mixtures. For this reason the larger number of experiments has been carried out with a partially hydrolyzed protein, commonly known as peptone, as substrate. Because it exhibits its maximum activity in the further hydrolysis of peptide nitrogen, this enzyme is referred to as peptonase, a term indicative of its action on peptone.

The intracellular peptonase of pneumococcus hydrolyzes 30 to 40 per cent of the peptide nitrogen in peptone substrates to amino nitrogen. The peptonase activity of the bacterial substance is striking in its intensity. Weight for weight the substance hydrolyzes peptone several times as rapidly as the most active commercial samples of pancreatic preparations. The zone of its optimum activity is pH 7 to 7.8, similar to that of trypsin and erepsin, and corresponds to the optimum reaction for growth of pneumococcus. The absence of activity at a pH below 4.5 indicates the absence of pepsin. Bile salts, as well as bile itself, effect solution of pneumococci, and enzymes prepared by dissolving the cell bodies in solutions of sodium choleate manifest an equal degree of activity. The thermostability of the intracellular peptonase is greater than the heat resistance of pneumococcus. The enzyme is, however, sensitive to heat; an exposure of 10 minutes at 100°C. destroys its activity. Dissolved in undiluted ox bile the enzyme retained about 40 per cent of its activity over a period of 6 weeks. A direct proportionality has been shown to exist between the rate of hydrolysis and the concentration of the enzyme in the digestion mixture.

In bacteria-free filtrates of pneumococcus enzymes are demonstrable only when growth of the bacteria has progressed to the phase in which cell disintegration begins and liberation of the intracellular sub-

stances into the culture medium occurs. During the early stages of growth of pneumococcus, when the organisms are multiplying at their maximum rate and little or no cell death is occurring, enzymes cannot be detected in culture filtrates. This evidence indicates that the enzymes studied are intracellular in character and belong to the class known as endoenzymes.

As far as is known for bacteria, solubility in bile is peculiar to pneumococcus alone. The mechanism of this reaction is not fully understood. Pneumococci exposed to an acidity equivalent to or greater than pH 5 are not only rapidly killed but rendered completely bile-insoluble. The endoenzymes derived from pneumococcus, on the other hand, are little influenced in their subsequent activity by previous exposure for 2 hours to a reaction corresponding to the acid death-point of the bacterial cell. Similarly pneumococci rapidly succumb on short exposure to a temperature of 52°C. and the heat-killed organisms are no longer soluble in bile. Exposure of the proteolytic enzyme, however, to a temperature corresponding to the thermal death-point of pneumococcus, causes only slight retardation of its hydrolyzing power.

These facts, apart from their significance in a study of the nature of the endoenzymes, are of interest in interpreting the possible relation of these active intracellular substances to the mechanism of bile solubility of pneumococcus. From these limited observations, it does not appear likely that bile solubility is the result of the action of enzymes, of which bile serves as an activator, for agents, both chemical and physical, which render the cell insoluble in bile, exert in a similar concentration only slight inhibition on the intracellular enzymes.

Rosenow found that extracts of virulent pneumococci possessed the power to split foreign proteins such as those present in ascites meat broth, while extracts of non-virulent organisms showed no digestion. The observations recorded in this paper on the relation of virulence to enzyme activity of pneumococcus are too limited to warrant any final judgment. However, under the experimental conditions, loss of virulence of the organism was not associated with a corresponding loss of enzyme activity. Pneumococci with virulence differing by a ratio of 1,000,000 to 1 showed quantitatively identical proteolytic power. In further elucidation of this problem it would be of interest

not merely to compare differences in enzymic activity with variations in virulence of the same strain, but also to contrast the relative potency of enzyme preparations from pathogenic pneumococci of the disease-producing types with the activity of similar preparations from the more saprophytic varieties of little or no virulence.

SUMMARY.

1. Pneumococci contain an intracellular enzyme or enzymes which (*a*) hydrolyze to some extent intact protein and (*b*) hydrolyze with striking avidity peptones. The optimum reaction for hydrolysis is pH 7 to 7.8, which also represents the optimum for the growth of pneumococcus. For convenience the terms "protease" and "peptonase" have been used, but no assumption is made as to whether the two actions, proteolysis and peptolysis, are due to two separate enzymes or are two activities of the same enzyme.

2. Solutions of intracellular substance of comparable enzymic activity may be prepared by dissolving the bacteria in bile, in sodium choleate, or by mechanical and autolytic disintegration of the cell.

3. The rapidity with which peptone is hydrolyzed is proportional to the concentration of the enzyme.

4. Heating the enzyme for 10 minutes at 100°C. destroys its activity.

5. Increasing the acidity to pH 5, the acid death-point of pneumococcus, suspends activity but does not destroy the enzyme, for activity is restored by readjustment to pH 7.8.

6. Attenuation of virulence to 1/1,000,000 of the original virulence had no measurable quantitative effect on the enzyme activity.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

II. Lipolytic Enzymes: Esterase.

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In the preceding paper (1) are recorded the facts so far obtained in a study of the proteolytic enzymes of pneumococcus. It has been shown that bile solutions of pneumococci, extracts obtained by disintegration of the organisms in phosphate mixtures, and sterile filtrates of autolyzing broth cultures possess the power to hydrolyze peptones and to a less extent certain intact proteins. In the present paper evidence will be presented that pneumococci possess also an endolipase of marked activity. The intracellular nature of this enzyme, the influence of age and hydrogen ion concentration on its activity, its thermostability, and relation to virulence and to the mechanism of bile solubility will be discussed.

In a monograph on bacterial enzymes, Fuhrmann (2) (1907) summarizes the work of earlier investigators on the occurrence of lipases in different species of microorganisms. In a review of the literature no reference has been found to work on the lipase of pneumococcus. Various methods for the demonstration of lipolytic activity of bacteria have been used. By a simple plate method, in which the zone of reaction about colonies of bacteria growing in the presence of certain test substances such as fat could be observed, Eijkman (3) detected lipolytic action in a number of organisms, including *Staphylococcus aureus*, *B. pyocyaneus*, *B. prodigiosus*, and *B. fluorescens*. Studying the lipase of *B. tuberculosis* and other bacteria, Wells and Corper (4), by testing the killed bacterial substance on esters and fats, demonstrated the presence of lipolytic enzymes in organisms which by the plate method apparently possessed no visible fat-splitting power. They further showed that sterile unheated emulsions of *B. tuberculosis*, while not actively lipolytic, possess enzymes capable of slowly hydrolyzing esters. Kendall, Walker, and Day (5) have demonstrated the occurrence of a soluble lipase in broth cultures of a variety of acid-fast organisms including tubercle bacilli of the human, bovine, and avian types. These authors found that the organisms, during the period of active growth, excrete a soluble lipase which occurs free in the medium.

Kendall and Simonds (6) have shown that sterile filtrates of plain and dextrose broth cultures of typhoid bacilli contain an esterase, capable of liberating acid from ethyl butyrate. The bacteria separated from the filtrates, however, showed but little esterase activity.

EXPERIMENTAL.

Methods.

Kastle and Loevenhart (7) have shown the advantage of using the lower esters, tributyrin and ethyl butyrate, in studying lipase activity. A preliminary experiment showed that when pneumococci are dissolved in bile the resultant solution contains an enzyme that splits both these esters. In the experiments to be recorded tributyrin has been used throughout as the substrate in the study of the intracellular lipase.

In determining lipase activity it has been customary to adjust the fat or ester substrate to approximate neutrality, with phenolphthalein as indicator, and then by titration to determine the amount of acid yielded by enzyme action. In the present study, however, it seemed more important to establish the optimum hydrogen ion concentration for action of the lipase, and then to maintain this reaction by the use of suitable buffer solutions. This buffered substrate maintains optimum conditions for enzyme action with a minimum of inhibition due to the acid products of hydrolysis. The amount of acid split off from the ester is determined by the amount of alkali required to readjust the digestion mixture to the initial reaction. It may also be calculated as the amount of acid required to change the buffered digestion mixture from the initial to the final hydrogen ion concentration. In the following experiments the ester was emulsified in 0.1 M phosphate solution of desired pH.

The method of preparing the enzyme solution, by dissolving the bacterial cells in sterile bile, was the same as that recorded in the experiments on the proteolytic enzymes of pneumococcus.

In no instances were antiseptics used as preservatives in the digestion mixtures. Sterility of all enzyme-substrate emulsions was proved by subculture.

Presence of an Intracellular Lipase and the Influence of Hydrogen Ion Concentration on Its Activity.

Experiment 1. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was washed in isotonic salt solution, taken up in 20 cc. of sterile, undiluted ox bile, and held over night in the ice box. A portion of this enzyme solution was inactivated by heat.

(*b*) *Preparation of Substrate.*—2 per cent tributyrin (Kahlbaum) was emulsified in 0.1 M phosphate solution covering the range of pH values 4.9 to 7.8.

In carrying out the experiment 0.2 cc. of tributyrin was added to 10 cc. of sterile phosphate solution at the indicated reaction, and the mixtures were shaken until a fine emulsion was obtained. Three tubes were prepared at each reaction. 1 cc. of active enzyme solution was added to the first tube; 1 cc. of the same solution inactivated by heat to the second; and to the third 1 cc. of the undiluted bile used in preparing the bacterial solution. The tubes were then placed at 37°C. for 72 hours. The results of these experiments are given in Table I and are represented graphically in Text-fig. 1.

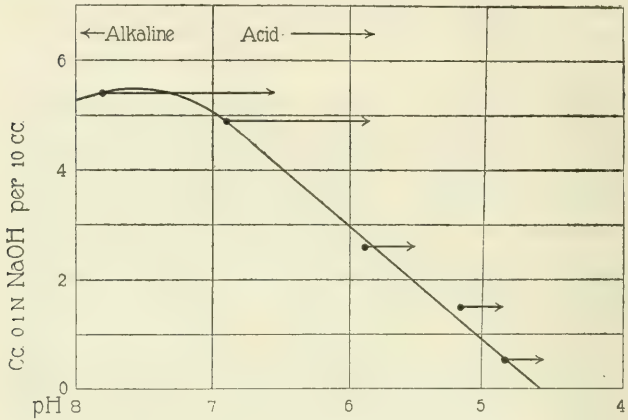
TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Lipase of Pneumococcus.

Initial reaction of digestion mixture.	Hydrogen ion concentration after 72 hrs. at 37°C.			10 cc. of mixture containing active enzyme readjusted with 0.1 N sodium hydroxide.	
	10 cc. of 2 per cent tributyrin + 1 cc. of				
	Bile.	Inactive enzyme.	Active enzyme.	To initial pH.	Amount required.
pH	*pH*	*pH*	*pH*	*pH*	*cc.*
4.9	4.9	4.9	4.6	4.9	0.3
5.2	5.2	5.2	4.9	5.2	1.5
5.9	5.9	5.9	5.5	5.9	2.3
6.9	6.9	6.9	5.8	6.9	4.9
7.8	7.8	7.8	6.5	7.8	5.4

The facts brought out in Experiment 1 demonstrate that within the pneumococcus cell there exists a markedly active lipase, or esterase. The acid formed from 10 cc. of 2 per cent tributyrin was equivalent to 5.4 cc. of 0.1 N alkali, or a normality of about 0.05 N butyric acid. The maximum activity of this esterase occurs at a reaction of about pH 7.8 and progressively decreases with increase in acidity. This

optimum reaction corresponds closely with that of the intracellular peptonase, and coincides with the optimum hydrogen ion concentration for growth of pneumococcus.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular lipase of pneumococcus. The arrows indicate the extent of the reaction change.

Intracellular Nature of the Pneumococcus Lipase.

In the preceding paper it was shown that in filtrates of broth cultures of pneumococcus proteolytic enzymes were demonstrable only in the later phases of growth. Their appearance free in the culture medium coincided with the dissolution of the bacterial cells. In the early stages of growth, however, when cell multiplication is occurring at a maximum rate no enzymes are demonstrable in the culture filtrate. These facts indicate the intracellular nature of the proteolytic enzyme. Similarly it is shown in the following experiment that during the early phases of growth no lipase is present in cell-free filtrates. That the organisms were actively growing is evidenced from the change in reaction of the culture from pH 7.8 to 7.4.

Experiment 2.—20 cc. of the Berkefeld filtrate of a 5 hour culture of No. F 208 (the same preparation that was used in Experiment 8 in the preceding paper) were divided into two 10 cc. portions, one of which was autoclaved; 0.1 cc. of tributyrin was then added to each and the tubes were incubated for 48 hours. The results are given in Table II.

TABLE II.

Absence of Lipase in Culture Filtrates of Pneumococcus during the Period of Active Growth.

Filtrates from a 5 hour broth culture of Pneumococcus Type II.

Sterile filtrate.	Tributyrin.	Hydrogen ion concentration of filtrate.	
		Before incubation.	After incubation.
cc.	*cc.*	*pH*	*pH*
10, unheated.	0.1	7.4	7.4
10, heated.	0.1	7.3	7.3

From Table II it is evident that the lipase, like the peptonase, is an intracellular substance liberated on disintegration of the bacterial cell.

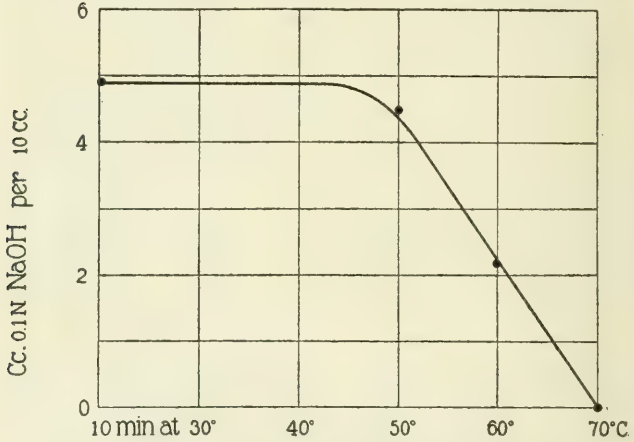
Effect of Heat on the Intracellular Lipase of Pneumococcus.

Experiment 3.—The thermostability of an enzyme in solution is influenced by the reaction of the medium in which it is dissolved and the length of exposure to the unfavorable temperature. In the present instance the enzyme was dissolved in bile, the solution adjusted to the optimum reaction for enzyme activity, pH 7.8, and subjected for 10 minutes in a water bath to the temperatures indicated.

The enzyme solution was the same as that used in Experiment 1 and was prepared from Pneumococcus Type II (No. F 208). 1 cc. portions of dissolved enzyme were carefully placed in sterile tubes and completely immersed in a water bath at the given temperature for 10 minutes. The tubes were immediately cooled to room temperature and to each were added 10 cc. of 2 per cent of tributyrin in 0.1 M phosphate solution, pH 7.8. After 24 hours at 37°C. the amount of acid split off from the ester by enzyme action was determined as in Experiment 1. The results are graphically presented in Text-fig. 2.

Variations in heat susceptibility of bacterial enzymes have been observed by many investigators. Söhngen (8), in studying the process of fat-splitting by bacteria, describes a lipase which resists a tempera-

ture of 100°C. for 5 minutes. The thermostability of the lipase of acid-fast bacteria has been noted by Wells and Corper, and by Kendall, Walker, and Day. These authors point out that heating to 100°C. for 15 minutes had little effect on the activity of the enzyme. Resistance of lipases in general to high temperatures is unusual; the various plant and tissue lipases in solution are as a rule inactivated by temperatures of 60–70°C. From Text-fig. 2 which illustrates the



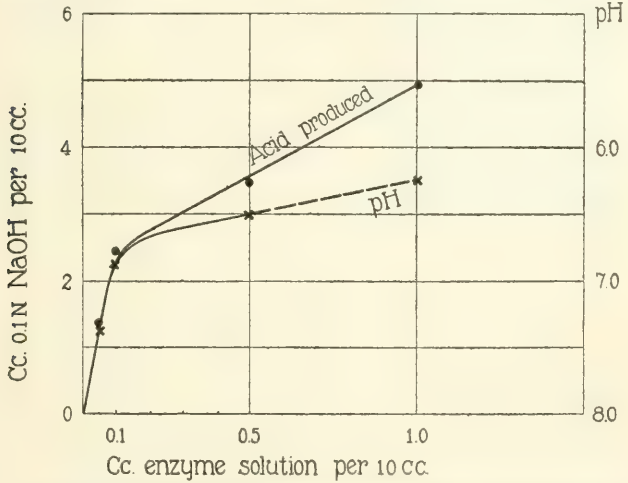
TEXT-FIG. 2. Heat stability of the intracellular lipase of pneumococcus.

effect on the pneumococcus lipase of exposure to various temperatures, it is evident that the dissolved enzyme suffers progressive loss of activity at temperatures above 50°C. and complete destruction at 70°C. for 10 minutes. According to Sternberg (9) the thermal death-point of pneumococcus is 52°C. for 10 minutes. The thermostability of the intracellular lipase under the conditions of this experiment, therefore, is somewhat greater than the heat resistance of the living organism.

Effect of Concentration of Enzyme on the Activity of the Intracellular Lipase of Pneumococcus.

If the ester-splitting property of bile solutions of pneumococci is enzymotic in nature, the rate of acid production should be proportional to the concentration of enzyme present. That this is the case is shown in the following experiment.

Experiment 4.—The enzyme solution, the same preparation used in the preceding experiment, was diluted with bile so that 1 cc. contained from 0.05 to 1 cc. of the original enzyme solution. These graduated quantities were added to 10 cc. of substrate consisting of 2 per cent tributyrin emulsified in 0.1 M phosphate solution of pH 7.8. The enzyme-substrate mixtures were incubated at 37°C. for 24 hours and the amount of acid hydrolysis was determined by the method outlined. The results are given in Text-fig. 3.



TEXT-FIG. 3. Influence of concentration of enzyme on the activity of the intracellular lipase of pneumococcus.

It is evident from the curves presented, that the rate of acid production by the action of the intracellular lipase on tributyrin is

directly proportional to the concentration of enzyme. Furthermore, as the enzyme concentration approaches the maximum, the amount of acid liberated by ester cleavage becomes so great that the resulting acidity is of itself sufficient to retard further enzyme action.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Experiment 5. Preparation of Enzyme.—Two subcultures of the same strain of Pneumococcus Type II (No. F 208) were used. One of these, designated No. F 208 "B," the virulence of which had been greatly attenuated by cultural methods, failed to kill white mice in doses of 1 cc. of broth culture. The other, No. F 208 A, representing the original strain, the virulence of which had been preserved, was invariably fatal to these animals in doses of 0.000001 cc. injected intraperitoneally. The washed bacterial residues from 1,500 cc. of 15 hour plain broth cultures of these two organisms were collected and each was dissolved in 15 cc. of undiluted, sterile ox bile. The respective solutions were held over night in the ice box to ensure complete plasmolysis and were then tested for sterility on blood agar and in broth. The control enzyme solutions were inactivated by heat.

1 cc. portions of the active and inactivated enzyme preparations were added to tubes containing 10 cc. of 2 per cent tributyrin emulsified in sterile 0.1 M phosphate solution at pH 7.8. The digestion mixtures were then incubated for 24 hours at 37°C. Determinations of the hydrogen ion concentrations before and after incubation and of the amounts of acid produced in each instance are given in Table III.

TABLE III.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Pneumococcus Type II.		Enzyme.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.	Increase due to enzyme action.
Strain.	Minimum lethal dose for mice.		Initial.	Final		
	cc.		*pH*	*pH*	*cc.*	*cc.*
F 208 "B"	Greater than 1	Active.	7.8	6.7	3.95	3.60
		Inactive.	7.8	7.7	0.35	
F 208 A	0.000001	Active.	7.8	6.7	3.75	3.45
		Inactive.	7.8	7.7	0.30	

Under the conditions of Experiment 5, in which were compared the relative potencies of the endoenzymes of an avirulent and of a virulent culture of the same strain of pneumococcus, loss of virulence was not associated with loss of enzyme activity.

Effect of Exposure to Acid Reaction on the Subsequent Activity of Pneumococcus Lipase.

Lord and Nye (10) have shown that pneumococci are rapidly killed at a hydrogen ion concentration of about pH 5.1. This acid deathpoint has been found both by these observers and by the present authors to correspond to the final reaction of broth cultures of pneumococcus when grown in the presence of sufficient carbohydrate. To determine whether this reaction is fatal to both organism and enzyme alike and whether this correlation has any significance in the mechanism of bile solubility the following experiment was performed.

Experiment 6.—1 cc. of active enzyme solution prepared as in Experiment 1 was added to 9 cc. of 0.1 M acid potassium phosphate solution of pH 4.6 (resulting reaction pH 5) and incubated at 37°C. for 2 hours. Then 0.83 cc. of N sodium hydroxide (calculated and verified on separate 9 cc. samples) was added to bring the acid enzyme solution to pH 7.8. After this readjustment of reaction 0.2 cc. of tributyrin was added as substrate, and the enzyme-substrate mixture incubated for 42 hours at 37°C. To serve as a control on activity, 1 cc. of the same enzyme solution, untreated, was added directly to 10 cc. of 0.1 M phosphate solution at pH 7.8 containing 2 per cent tributyrin (Table IV).

TABLE IV.

Effect on Lipase of Exposure to Acid Reaction.

Pneumococcus Type II.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
	Initial.	Final.	
	pH	*pH*	*cc*
Untreated enzyme	7.8	6.3	4.93
Acid-treated and readjusted	7.8	6.4	3.80

From Table IV it is evident that after neutralization the activity of the pneumococcus lipase is little influenced by previous exposure

to a reaction of pH 5. The bile insolubility of pneumococci after similar acid treatment is apparently not attributable to the death of the lipase, but is possibly referable to changes in the cell protoplasm.

Effect of Age on the Activity of the Intracellular Lipase of Pneumococcus.

Experiment 7.—Enzyme solutions prepared from pneumococci by extraction in phosphate solution as described in the preceding paper possessed lipase activity entirely comparable to that of enzyme solutions prepared by the bile method. These preparations were still active after preservation for 7 weeks, as is shown in Table V.

TABLE V.

Age Stability of Lipase.

Pneumococcus Type II.	Age.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
		Initial (0.1 M phosphate).	Final.	
	wks.	*pH*	*pH*	*cc.*
No. F 208	7	7.8	7.1	4.9
" II	3	7.8	6.9	6.5

DISCUSSION.

When pneumococci are dissolved in bile or extracted by the methods described, the resultant solution possesses, in addition to the proteolytic activity recorded in the preceding paper, a lipase (esterase) as measured by its power to split off acid from tributyrin.

Since a number of investigators (Hewlett (11), Magnus (12), and Loevenhart and Souder (13)) have shown that bile and bile salts not only do not interfere with lipase activity, but on the contrary accelerate the reaction, it was to be expected that the use of bile in effecting solutions of pneumococci would serve as an ideal method for demonstrating lipase activity. Bile, however, is not essential to the reaction, since extracts prepared by other methods exhibit comparable activity.

This esterase manifests its maximum activity at a hydrogen ion concentration of about pH 7.8, which is the optimum reaction for initiating growth of pneumococcus. The lipolytic activity of this enzyme progressively diminishes with increasing acidity until at about

pH 5 further hydrolysis ceases. That the point of acid extinction of esterase activity corresponds closely with the acid death-point of the living pneumococcus reveals another interesting correlation between cellular function and enzyme action. The rate of acid liberation from tributyrin during the initial stage of the action of the intracellular lipase is directly proportional to the concentration of enzyme. In the later phases of the reaction, the acid liberated by ester cleavage is sufficient in itself to retard further hydrolysis.

That the pneumococcus lipase is intracellular in nature is evidenced by the fact that it is present in maximum amount in bile solutions of washed bacterial cells, but cannot be demonstrated in culture filtrates during the period of active growth of the organism. The thermostability of the endolipase in solution is greater than the heat resistance of the living pneumococcus. After 10 minutes exposure in a water bath at temperatures greater than 50°C., the dissolved enzyme suffers progressive loss in activity until at 70°C. complete destruction results. Enzyme solutions preserved at refrigerator temperature retain their activity for weeks.

As to the possible relation of the activity of endoenzymes of pneumococcus to virulence of the living cell, the observations recorded in these studies are too limited to warrant discussion. Comparison of the relative potency of the endoenzymes of avirulent and virulent cultures of the same strain of pneumococcus has shown, however, that under the experimental conditions defined, loss of virulence was not associated with loss of enzymic activity.

Pneumococci exposed to a reaction corresponding to the acid death-point of the cell, that is, an acidity equivalent to or greater than pH 5, are thereby rendered insoluble in bile. This bile insolubility of pneumococci after acid treatment persists upon neutralization of the acid and even after the cells have been removed, washed, and resuspended in a neutral medium. In view of the possibility that the mechanism of bile solubility might in some way be related to the endolipase, in which instance the bile salts might function as coenzyme, or activator, it seemed pertinent to determine the effect of exposure to acid reaction on the subsequent activity of the enzyme itself. Since it has been shown, however, that upon neutralization the activity of the lipase is little influenced by previous exposure to a reaction of

pH 5, an acidity which kills the living cell, the phenomenon of bile insolubility of pneumococci after similar acid treatment is apparently not attributable to destruction of the endoenzyme.

SUMMARY.

1. Pneumococci contain an intracellular enzyme of marked lipolytic activity as measured by the acid liberated by its action on tributyrin.

2. Enzyme-containing solutions may be prepared by dissolving pneumococci in bile, or by extraction by other means.

3. The optimum reaction for maximum activity of the endolipase is about pH 7.8, which coincides with the optimum hydrogen ion concentration for growth of pneumococci.

4. Heating the enzyme for 10 minutes at 70°C. destroys its activity.

5. Attenuation of virulence of pneumococcus had no measureable effect on enzyme activity.

6. The possible relation of the endolipase to the mechanism of bile solubility is discussed.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

III. Carbohydrate-Splitting Enzymes: Invertase, Amylase, and Inulase.

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The two preceding papers (1, 2) on the enzymes of pneumococcus have dealt with the proteolytic and lipolytic activities of extracts of the bacterial cells, and of sterile filtrates of cultures. It has been pointed out that enzymes capable of actively hydrolyzing various substrates exist preformed within the cell and that by suitable methods they can be obtained free in solution and their action studied independently of the living organism.

The avidity with which pneumococcus attacks certain carbohydrates is manifest in the accelerated growth and increased acid production of organisms cultivated in the presence of these substances. Acids are produced in culture media from starches and glucosides, as well as from mono- and disaccharides. It has been customary to assume that these fermentation reactions are the summation of the action of several enzymes, first, the hydrolysis of the disaccharide or starch to monosaccharide, and secondly, the production of acid from the monosaccharide.

It was of interest, therefore, to determine whether the bile solutions of pneumococci which were known to contain protein- and fat-splitting enzymes also contained either or both groups of carbohydrate enzymes. Tests upon glucose, saccharose, starch, and inulin substrates were carried out with enzyme solutions containing peptonase and lipase prepared by dissolving pneumococci in bile.

The simple expedient of dissolving the bacterial cells in bile and testing the resultant solution for the presence of enzymes, a method admirably adapted to the determination of proteolytic and lipolytic activity, was found unsatisfactory for studying the enzymes attacking

carbohydrates, since bile in the concentration necessary to effect bacterial solution completely inhibits the activity of the enzymes converting sugar and starch. Consequently the method described below was adopted, by which disintegration of the pneumococcal cells is effected by suspending them in balanced phosphate solution at pH 6.2 and hastening physical disruption by repeated freezing and thawing of the bacterial suspension. The enzymes liberated from the bacterial bodies in this manner, as will be shown in the following experiments, are capable of hydrolyzing sucrose, starch, and inulin, the three test substances chosen for the typical reactions of invertase, amylase, and inulase.

EXPERIMENTAL.

Bacteriological Methods.

Preparation of Enzyme Solution by the Acetone Method.—Because of the fact that precipitation with acetone has been a satisfactory method for preparing and purifying many types of enzymes, an attempt was made to obtain an active carbohydrate-splitting enzyme from pneumococcus by the following procedure.

The residue of 2 liters of an 18 hour broth culture of pneumococcus was taken up in two 10 cc. portions of sterile distilled water, and each portion was poured into 150 cc. of acetone. After standing over night the supernatant solutions were removed from the precipitates which were then allowed to dry. Cultural examination showed that the organisms had been killed by acetone precipitation, although they remained intact and were Gram-positive.

One portion of the dry residue was shaken up with 10 cc. of 0.1 M phosphate at pH 7.4; the other was treated with 2 cc. of 0.1 N sodium hydroxide, allowed to stand for several hours, then neutralized with 0.1 N hydrochloric acid to pH 7.4, and diluted to 10 cc. Each portion was tested for lipase, for peptonase, and for invertase. The tests were all negative.

Preparation of Enzyme Solution at pH 6.2.—Previous work has shown that growth of pneumococcus cannot be initiated at a reaction more acid than pH 6.8 (3), and that disintegration of the bacterial

cell occurs most rapidly at about pH 6.2. Moreover, although the subsequent activity of the intracellular lipase and peptonase at optimum reactions is not materially influenced by temporary exposure to an acidity as great as pH 5, they show much less activity at pH 6.2 than at a neutral or slightly alkaline reaction. It seemed probable therefore, that disintegration of the pneumococcus cell, under conditions at which the autolytic processes are at a minimum and at which neither initial growth of the organism nor destruction of known enzymes occurs, might liberate the intracellular carbohydrate-splitting enzymes. Such conditions were obtained in the following manner.

The washed bacterial residue from 1.5 liters of an 18 hour plain broth culture of pneumococcus was taken up in 15 cc. of 0.1 M phosphate solution of pH 6.2, and placed in the ice box until intact bacterial cells could no longer be found under the microscope. After the solution had been proved sterile, it was tested for the presence of known enzymes. The lipase activity of this solution was comparable to that obtained with the bile solution previously used.

Preparation of Enzyme Solution by Cytolysis with Alternate Freezing and Thawing.—This method differed from the preceding only in that the disintegration of the cell was hastened by repeated freezing and thawing. An ice-salt mixture of about −22°C. was used.

Sterility Controls.—In addition to the sterility tests on the enzyme solution, the final enzyme-substrate mixtures were tested by transferring 0.1 cc. to 5 cc. of broth and incubating for 48 hours.

Chemical Methods.

Preparation of Substrates. Sucrose.—A 4 per cent sucrose solution was sterilized in boiling water for 20 minutes. 25 cc. of this sterile 4 per cent sugar solution were then added to 25 cc. of sterile phosphate solution of the desired pH.

Glucose.—Prepared in the same manner as sucrose.

Starch.—A 2 per cent and a 0.2 per cent suspension of Kahlbaum's rice starch in 0.1 M phosphate solution of pH 7.4 were autoclaved for 20 minutes at 15 pounds pressure.

Inulin.—A 2 per cent inulin solution in 0.1 M phosphate at pH 7.4 was sterilized as above.

Phosphate Solutions.—The 0.1 M phosphate solutions for the range pH 5 to 8.3 were prepared from potassium acid phosphate and sodium phosphate (Na_2HPO_4) according to Sörensen's tables. For the more acid range, mixtures of 0.1 M potassium acid phosphate and 0.1 N hydrochloric acid were used. In all the experiments the appropriate corrections were made for the effect on the reaction of the enzyme solution. All phosphate solutions were sterilized by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The hydrogen ion concentration was ordinarily determined by the colorimetric method. For the range of acidity greater than pH 4.5 and for frequent controls of the colorimetric standards, the electrometric method, using Clark's rocking electrodes (4), was employed.

Determination of Reducing Sugar.—Qualitative tests for reducing sugar were carried out by using 5 cc. of Benedict's qualitative solution and 0.5 cc. of test solution and boiling for 10 minutes.

Quantitative sugar determinations were made with either Benedict's quantitative titrating method, by determining the rotation of the solution, or by the gravimetric copper method.

Carbon Dioxide.—Determinations were made with Van Slyke's apparatus (5).

Amylase Action.—Hydrolysis of starch to dextrins was determined by means of the iodine color test. 1 cc. of the solution was diluted with 2 cc. of water and 0.2 cc. of a dilute iodine solution (about $\frac{N}{150}$) added. Hydrolysis of the starch or dextrin to reducing sugar was determined as indicated above.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Experiment 1.—A solution of Pneumococcus Type II prepared as outlined above was added to a series of tubes containing saccharose, inulin, starch, glucose, and glucose-peptone mixture. The glucose-peptone solution was used with the idea that the nitrogen of the peptone might accelerate glucose hydrolysis. The tubes were incubated for 48 hours at 37°C. The solutions were then analyzed as described. The results are given in Table I.

Experiment 2.—This experiment differed from the preceding one in that the suspension of pneumococci was frozen and thawed five times. The enzyme solution was held at ice box temperature (4°C.) for 16 days until culture controls in blood broth no longer showed the presence of viable organisms.

In addition to the substrates used in the preceding experiment two additional control tubes were included, one containing 10 cc. of a 2 per cent saccharose solution in 0.1 M phosphate solution plus 1 cc. of bile, and the second containing 0.2 per cent glucose. Since slight glucose fermentation might have been masked by an excess of the sugar, the dilute solution was included in this series. The results are presented in Table II.

TABLE I.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by cytolysis at pH 6.2.

Substrate in 0.1 M phosphate solution.	Final hydrogen ion concentration.		Qualitative determinations with Benedict's solution.		Color with iodine.		Rotation.		Carbon dioxide content per 2 cc.	
	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.
	pH	*pH*							*cc.*	*cc.*
Saccharose, 2 per cent.	7.4	7.4	−	++++			2.7°	1.36°	0.075	0.080
Glucose, 2 per cent.	7.4	7.4	++++	++++					0.075	0.075
Glucose, 1 per cent, in 1 per cent peptone.	7.4	7.4	++++	++++					0.075	0.090
Inulin, 2 per cent.	7.4	7.4	−	++						
Starch, 2 per cent.	7.4	7.4	−	+	Blue.	Lavender.	0°	0.2°		
Starch, 0.2 per cent.	7.4	7.4	−	+	"	Vanishing light lavender.				
Tributyrin,* 1 per cent.	7.8	6.3								

* Tributyrin was used as a control of enzyme activity.

It is evident from Tables I and II that pneumococcus contains enzymes that hydrolyze starch to dextrins (amylase), hydrolyze inulin (inulase), and invert saccharose to reducing sugars (invertase). The microorganism appears, therefore, to contain enzymes capable of hydrolyzing complex carbohydrates into simple sugars. Bile

completely inhibits the hydrolysis; whether this is due to destruction of the enzyme or to inhibition of its action has not been determined. This fact explains the failure to detect the carbohydrate enzymes in the bile solution used for the study of the peptonase and lipase of pneumococcus. On the other hand, the peptonase and lipase are as active in the solution obtained by the method described as in the bile solutions.

All attempts to demonstrate an enzyme capable of fermenting glucose or producing acid from glucose were unsuccessful.

TABLE II.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by alternate freezing and thawing.

Substrate in 0.1 M phosphate solution.	Hydrogen ion concentration.		Qualitative determinations with Benedict's solution.	Color with iodine.	Carbon dioxide content per 2 cc.
	Initial.	Final.			
	pH	*pH*			*cc.*
Glucose, 2 per cent	7.3	7.3	++++		0.09
" 0.2 per cent	7.3	7.3	+++		
Starch, 2 per cent	7.3	7.3	++	Lavender.	
" 0.2 per cent	7.3	7.3	+	Vanishing lavender.	0.07
" 0.2 " " (control)	7.3	7.3	−	Blue.	
Inulin, 2 per cent	7.3	7.3	+		
Saccharose, 2 per cent	7.3	7.3	++++		0.07
" + 1 cc. of bile	7.3	7.3	−		
Control	7.3	7.3	−		
Tributyrin,* 1 per cent	7.8	6.3			

* To control enzyme activity.

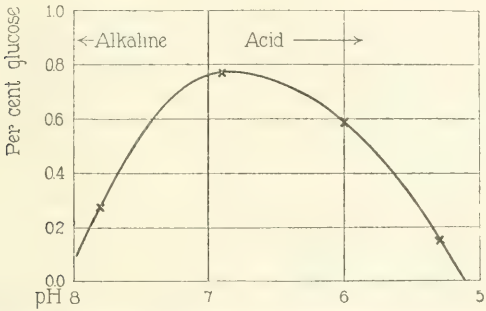
Intracellular Nature of the Carbohydrate-Splitting Enzymes.

Experiment 3.—Tests for invertase, amylase, and for a glucose-fermenting enzyme in the filtrate from a young actively growing culture of pneumococcus were carried out exactly as in the case of lipase and peptonase. Saccharose, starch, and glucose to make 1 per cent solutions were added to the sterile filtrate from a 5 hour culture of Pneumococcus Type II, and the mixture was incubated for 48 hours at 37°C. The mixtures were tested as in the preceding experiment and no evidence of enzyme action was demonstrable. Since in the solutions of pneumococci

obtained by the method described there are active carbohydrate-splitting enzymes, their absence in the filtrates of cultures during the early phase of growth, before cell disintegration has occurred, indicates that, like lipase and peptonase, these enzymes are intracellular in nature.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Invertase of Pneumococcus.

The preceding experiments have demonstrated the presence of several carbohydrate-splitting enzymes. It now seemed desirable to determine the influence of the hydrogen ion concentration on their activity.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular invertase of pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme Solution.*—The solution was prepared as described in preceding experiments.

(*b*) *Preparation of Substrate.*—The substrates were prepared as outlined under Methods. 1 cc. of enzyme was added to 10 cc. of substrate solution; a duplicate tube without enzyme served as control for acid hydrolysis.

Both qualitative and quantitative reducing sugar determinations were made. The results are given in Table III and Text-fig. 1.

Optimum Hydrogen Ion Concentration for Amylase Action.

These experiments were carried out as in the case of invertase. Although quantitative results were not carried out, the iodine color indicates that the optimum reaction is about pH 7.

TABLE III.

Influence of Reaction on Invertase Action.

Enzyme preparation.	Hydrogen ion concentration.	Glucose determinations.		
		Qualitative determinations with Benedict's solution.	Quantitative determinations by Benedict's method.	Quantitative determinations by the gravimetric copper method.
	pH		*per cent*	*per cent*
No. D 39	7.7	++	0.1	
" D 39	6.9	+++	0.51	
" D 39	6.1	+++	0.34	
" D 39	5.2	+	0.1	
" D 39	4.8	−	0.0	
" F 208	7.8	+±		0.29
" F 208	6.9	+++		0.77
" F 208	6.0	++		0.59
" F 208	5.3	+		0.15
" F 208	4.85	−		

All controls negative.

TABLE IV.

Influence of Reaction on Amylase Action.

Enzyme. preparation.	Hydrogen ion concentration.	Qualitative determinations with Benedict's solution.	Color with iodine.
	pH		
No. D 39	8.2	−	Deep, fading blue.
" D 39	6.9	++	Red-lavender.
" D 39	6.1	+	Blue-lavender.
" D 39	5.1	−	Blue.
" D 39	4.8	−	
" F 208	7.8	±	Very pale lavender, fading instantly.
" F 208	6.9	++	Pale lavender, fading rapidly.
" F 208	6.0	++	Deep " " slowly.
" F 208	5.3	−	Faint change only.
" F 208	4.8	−	Blue as controls.

It is evident from Tables III and IV that the optimum hydrogen ion concentration for pneumococcus invertase and amylase is about pH 7.

DISCUSSION.

It is generally accepted that in the utilization of carbohydrates by living bacteria, hydrolysis of these complex substances is brought about through the action of enzymes. The fact has been recognized that enzymes capable of converting sucrose and starch may be found in fungi, especially in yeasts and moulds. Studies on the carbohydrate-splitting processes of invertase-producing bacteria have also been reported by numerous investigators. The earlier work of Fermi and Montesano (3), particularly, lists a number of microorganisms, in sterile cultures of which invertase activity was demonstrable. The isolation and study of carbohydrate-splitting enzymes apart from the living cell have not, so far as we have been able to find in the literature, been attempted in the case of pneumococcus.

The demonstration of the intracellular agents concerned in carbohydrate cleavage by pneumococcus was accomplished by a method through which the release of endoenzymes from the intact organism was effected by breaking down of the cell structure under conditions not injurious to the reactive substances themselves. That physical disruption of the cell membrane through alternate freezing and thawing is subsequently followed by autolytic processes is indeed likely; that the chemical changes brought about by autolysis under these conditions, however, exert but slight influence on the activity of the enzymes studied is evidenced by the avidity with which hydrolysis occurs, and the length of time during which potency is preserved.

From the data presented in this and the two preceding papers, it may be concluded that within the cell bodies of pneumococci there exist in addition to the endohemotoxin described by Cole (6), a series of intracellular enzymes. The proteolytic and lipolytic functions of this endoenzyme-complex have already been described. In addition, there may now be added the activity of the endoenzymes causing hydrolysis of carbohydrate substances, such as sucrose, starch, and inulin.

The optimum hydrogen ion concentration for the invertase and amylase of pneumococcus is about pH 6.8 to 7. This represents a reaction slightly less alkaline than that shown to be optimum for the activity of the peptonase and esterase. The reaction most favorable

for the activity of enzymes that attack carbohydrates is not the same even for those having similar activities. Sörensen (7) has shown that the optimum hydrogen ion concentration for the invertase of beer yeast is about pH 4.5. In studying the influence of hydrogen ion concentration on the enzymic activity of three typical amylases of different origin, Sherman, Thomas, and Baldwin (8) found that the starch-splitting enzymes of malt and *Aspergillus oryzæ* are both most active at an acid reaction (pH 4.4 to 4.8), while the pancreatic amylase reaches its maximum at pH 7. The carbohydrate-splitting enzymes of pneumococcus function best at a neutral reaction and are operative over a zone which corresponds closely to the reaction range of the living organism when grown in the presence of fermentable substances (9). Attempts to determine the presence of an enzyme or enzymes capable of fermenting dextrose and producing acid, an action characteristic of the growing cell, have been unsuccessful.

The invertase, amylase, and inulase of pneumococcus, like the proteolytic and lipolytic enzymes, are intracellular in nature and are found free in culture fluids only after cell disintegration has begun.

SUMMARY.

1. A method is described for the preparation of an active enzyme-containing solution of pneumococci, in which no living cells are present. These enzymes are capable of hydrolyzing sucrose, starch, and inulin.

2. The invertase and amylase of pneumococcus are active within the limits pH 5 to 8, with an optimum reaction of about pH 7. This reaction range corresponds closely with limiting hydrogen ion concentrations which define growth of the organism in the presence of carbohydrate.

3. These studies indicate that the enzymes described are not true secretory products of the living cell, but are of the nature of endoenzymes, since their activity can be demonstrated only when cell disintegration has occurred.

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EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

III. The Permeability of Blood Corpuscles to Sugar.

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The subject of the permeability of the blood corpuscles to sugar was reviewed in 1913 by Allen[1] and in 1917 by Gradwohl and Blaivas.[2] Analyses covering both plasma and corpuscle sugar during glucose tolerance tests in human subjects were made by Bailey.[3] A large number of comparative analyses upon diabetic patients have already been published.[4] According to some statements in the literature, the corpuscles sometimes contain little or no sugar; according to others, their sugar content does not differ greatly from that of the plasma; and another belief has been that the corpuscles take up sugar more slowly and retain it longer, so that their sugar content is low in the early stages of hyperglycemia but above that of the plasma in the declining stages. The preponderance of experience is that the sugar content of the corpuscles is usually a little below that of the plasma.

The present investigation was carried out as part of an inquiry into the permeability of body structures for sugar, with a special view to any changes occurring in diabetes. Several other physiological and pathological conditions were considered, also the possible differences between species and between permeability *in vivo* and *in vitro*. Numerous analyses of the corpuscle mass after thorough centrifugation

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were performed but these were rejected in favor of calculations based on comparative analyses of whole blood and plasma. The method of analysis was that of Lewis and Benedict.[5] The reasons for the usual lack of strict agreement between the analyzed and calculated corpuscle sugars and for giving preference to the latter are obvious.

TABLE I.

Normal Dogs Receiving Glucose by Stomach.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Dog B2-98. Weight 4.3 kg. Given 9 gm. glucose per kg. by stomach tube in 30 per cent solution.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.062	0.079	0.026	3.04	72	32	Blood before feeding.
13	Slight.	0.159	0.232	0.017	13.64	70	34	½ hr. after "
5	0.22	0.345	0.371	0.274	1.35	63	27	1¼ hrs. " "
14	Faint.	0.116	0.125	0.086	1.45	45	23	1½ " " "
Dog C3-22. Weight 36 kg. Given 15 gm. glucose per kg. by stomach tube in 54 per cent solution.								
	0	0.089	0.111	0.035	3.17	71	29	Blood before feeding. Temperature 101.4° F.
16	Faint.	0.161	0.222	0.043	5.16	87	34	½ hr. after feeding. Temperature 101.1° F.
13	0.15	0.147	0.159	0.124	1.28	86	35	1 hr. after feeding. Temperature 101.0° F.
39	0.63	0.156	0.175	0.122	1.43	85	36	2½ hrs. after feeding. Temperature 100.6° F.
41	0.48	0.179	0.208	0.127	1.63	110	36	3¾ hrs. after feeding. Temperature 100.5° F.

Attention may be directed also to the necessity of precautions for complete precipitation of protein when dealing with corpuscles or even whole blood. Traces of unprecipitated protein may have an action upon either copper or picric solutions, and such small slips of technique are probably the commonest explanation of results which

[5] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

show the sugar concentration in the corpuscles as high as or higher than that in the plasma. Even with the greatest care in all particulars, any long series will show occasional discrepancies in the ratio of corpuscle sugar to plasma sugar which probably represent analytical errors.

Tables I and II show the results at various intervals after enteral and parenteral doses of glucose in normal dogs. The corpuscle sugar

TABLE II.

Normal Dogs Receiving Glucose Parenterally.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Dog C3-07. Weight 14.3 kg. Given 4 gm. glucose per kg. subcutaneously in 30 per cent solution.								
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.096	0.100	0.087	1.14	64	31	Blood before injection.
164	0	0.094	0.099	0.083	1.19	45	33	$18\frac{3}{4}$ hrs. after injection. Fed regular diet.
118	0	0.133	0.139	0.114	1.21	50	24	$26\frac{1}{2}$ hrs. after injection.
1,279	0	0.115	0.177	0.109	1.07	54	26	42 " " "
Dog C3-36. Weight 17 kg. Given 6 gm. glucose per kg. intraperitoneally and 4 gm. per kg. subcutaneously in 30 per cent solution. Total duration of injections 25 min.								
		0.093	0.115	0.063	1.82	114	43	Blood before injection.
		0.286	1.000	0.160	6.25	148		5 min. after end of injection.
		0.578		0.143*		122		25 min. after end of injection.

* Determined.

is regularly below that of the plasma, both during the rise and during the decline of hyperglycemia; and sometimes, as in Dogs B2–98 and C3-22, the difference is extreme.

Tables III to VI likewise illustrate the effect of glucose by stomach or subcutaneously in dogs depancreatized short of diabetes. There are minor differences between individual dogs but no perceptible

change of corpuscle permeability due to the operation. Control doses of plain saline also gave no change. This series included Dog B2-01, which was characterized by a noticeably low renal threshold for sugar,[6] but the corpuscles appeared no more permeable than in other dogs.

TABLE III.

Dog B2-60.

After removal of only the splenic process of the pancreas. Weight 45 kg.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
			4 gm. glucose per kg. by stomach tube in 30 per cent solution.					
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.133	0.136	0.127	1.07	40	34	Blood before feeding.
70	0.76	0.200	0.278	0.042	6.61	52	33	2½ hrs. after "
260	0	0.095	0.105	0.073	1.44	60	32	10½ " " "
35	0	0.088	0.093	0.076	1.22	49	30	23 " " "
			4 gm. glucose per kg. subcutaneously in 30 per cent solution.					
	0	0.086	0.081	0.097	0.83	54	31	Blood before injection.
55	Slight.	0.125	0.135	0.113	1.19	96	46	6 hrs. after "
65	0.26	0.103	0.143	0.056	2.55	88	46	23 " " "
			600 cc. 0.85 per cent NaCl solution subcutaneously.					
	0	0.105	0.105	0.105	1.00	71	41	Blood before injection.
45	0	0.117	0.117	0.117	1.00	75	35	3 hrs. after "
92	0	0.100	0.105	0.088	1.19	60	30	5½ " " "
312	0	0.091	0.110	0.058	1.89	77	37	24 " " "

[6] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xlii, 420, 439.

TABLE IV.

Dog B2-61.

After removal of $\frac{3}{4}$ of the pancreas. Weight 6 kg.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
								3 gm. glucose per kg. in 30 per cent solution by stomach tube.
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.122	0.143	0.090	1.59	87	40	Blood before feeding.
50	0.32	0.105	0.112	0.103	1.09	79	60	3 hrs. after "
50	0	0.100	0.111	0.080	1.39	80	36	$10\frac{1}{2}$ " " "
10	0	0.095	0.011	0.057	1.89	51	30	22 " " "
								3 gm. glucose per kg. in 30 per cent solution subcutaneously.
	0	0.105	0.106	0.104	1.02	110	49	Blood before injection.
7	Slight.	0.118	0.147	0.079	1.86	120	43	$3\frac{1}{2}$ hrs. after "
21	Faint.	0.111	0.111	0.111	1.00	95	44	22 " " "
								50 cc. 0.85 per cent NaCl solution subcutaneously.
	0	0.122	0.128	0.110	1.16	63	35	Blood before injection.
25	0	0.105	0.118	0.081	1.45	77	36	$3\frac{1}{2}$ hrs. after "
								4 gm. glucose per kg. in 30 per cent solution subcutaneously.
	0	0.105	0.133	0.053	2.51	65	35	Blood before injection.
56	Faint.	0.100	0.109	0.080	1.36	91	34	18 hrs. after injection. Fed regular diet.
35	0	0.161	0.167	0.147	1.13	65	30	26 hrs. after injection.
175	0	0.114	0.117	0.109	1.07	76	39	42 " " "

TABLE V.

Dog B2-00.

Weight 14 kg. Depancreatized not quite to point of diabetes.
3 gm. glucose per kg. in 30 per cent solution by stomach tube.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.122	0.122	0.122	1.00	52	36	Blood before feeding.
0	0.161	0.238	0.115	2.07	58	34	1 hr. after "
0	0.100	0.110	0.080	1.37	65	34	8 hrs. " "
0	0.096	0.094	0.100	0.94	61	29	10 " " "
0	0.091	0.091	0.091	1.00	58	28	15 " " "
0	0.078	0.110	0.028	3.93	71	39	24 " " "
0	0.105	0.111	0.094	1.18	58	35	27 " " "
0	0.113	0.118	0.102	1.15	60	32	29½ " " "
0	0.102	0.108	0.089	1.21	51	32	32 " " "

3 gm. glucose per kg. in 30 per cent solution subcutaneously.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Volume.	Glucose.							
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.065	0.065	0.065	1.00	100	40	Blood before injection.
	Slight.	0.134	0.163	0.103	1.58	105	49	2¾ hrs. after "
	0.69	0.134	0.154	0.115	1.34	120	52	8 " " "
	0.82	0.098	0.109	0.086	1.26	100	49	11 " " "
	Faint.	0.100	0.118	0.075	1.57	97	42	23½ " " "

140 cc. 0.85 per cent NaCl solution subcutaneously.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.114	0.116	0.110	1.05	59	34	Before injection.
Faint.	0.105	0.114	0.085	1.34	58	32	1 hr. after injection.
0	0.100	0.130	0.036	3.61	67	32	3½ hrs. " "
0	0.128	0.130	0.122	1.06	40	27	6½ " " "
0	0.112	0.118	0.097	1.21	58	29	10 " " "
0	0.100	0.102	0.095	1.07	57	29	23½ " " "

TABLE VI.

Dog B2-01.

Weight 14 kg. Depancreatized not quite to point of diabetes.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
			4 gm. glucose per kg. in 30 per cent solution by stomach tube.				
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.090	0.116	0.068	1.70	109		Blood before feeding.
Faint.	0.179	0.192	0.164	1.17	114	47	1 hr. after "
	0.100	0.106	0.092	1.15	100	45	3 hrs. " "
0	0.105	0.131	0.079	1.65	104	50	7 " " "
0	0.089	0.132	0.029	4.55	101	42	24 " " "
			4 gm. glucose per kg. in 30 per cent solution subcutaneously.				
	0.099	0.172	0.034	5.06	105	53	Blood before injection.
	0.166	0.244	0.096	2.54	124	53	½ hr. after "
	0.192	0.266	0.115	2.31	104	49	2 hrs. " "
	0.217	0.284	0.160	1.77	107	54	5½ " " "
	0.200	0.324	0.080	4.05	106	51	7 " " "
	0.166	0.230	0.121	1.90	108	59	9¾ " " "
	0.151	0.186	0.108	1.72	100	45	12 " " "
	0.158	0.163	0.151	1.08	97	43	14 " " "
	0.100	0.146	0.041	3.56	100	44	25 " " "

TABLE VII.

Dog B2-02.

Weight 10.5 kg. Very mild diabetes.

3 gm. glucose per kg. in 30 per cent solution by stomach tube.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.154	0.244	0.019	12.84	80	40	Blood before feeding.
4.50	0.196	0.270	0.080	3.37	82	39	1 hr. after "
1.85	0.149	0.159	0.133	1.12	77	38	2¾ hrs. " "
Very faint.	0.093	0.112	0.062	1.80	75	38	4 " " "
0	0.094	0.112	0.067	1.67	80	40	7 " " "

The same conclusions hold for the mildly diabetic (Tables VII and VIII) and the severely diabetic (Tables IX and X) dogs. The last mentioned animal was found[6] in the preceding paper to have a very high renal threshold, but no comparable change in the permeability of the corpuscles for sugar was demonstrable.

TABLE VIII.

Mildly Diabetic Dogs on Starch Feeding.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
Dog B2-43. Weight 10 kg. July 19, 1915.							
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0		0.133	0.103	1.29	70	43.5	Blood before feeding 100 gm. bread.
Faint.	0.137	0.178	0.087	2.04	96	45.0	2 hrs. after feeding.
0.53	0.189	0.222	0.133	1.66		37.1	5 " " "
July 23.							
0	0.116	0.125	0.100	1.25	96	37.3	Blood before feeding 100 gm. bread.
0.98	0.172	0.217	0.095	2.28	93	37.0	3 hrs. after feeding.
Dog B2-71. Weight 14 kg.							
0		0.125	0.112	1.11	90	27.2	Before feeding 200 gm. rice.
0.29	0.213	0.286	0.100	2.86	72		2 hrs. after feeding.
1.47	0.244	0.286	0.157	1.82		32.5	5 " " "

Table X gives the results of direct analyses of the corpuscles in comparison with the plasma after fat feeding in a severely diabetic animal. Though marked lipemia occurred, there was no appreciable alteration in the distribution of sugar between plasma and corpuscles.

TABLE IX.

Dog B2-79.

Weight 14.7 kg. Severe diabetes. 5 gm. glucose per kg. in 80 per cent solution by stomach tube.

Urine.								Remarks.
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar.	Hemoglobin.	Corpuscle volume.	
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.105	0.105	0.105	1.00	108	47	Blood before injection.
6	Slight.	0.152	0.182	0.117	1.55	100	46	½ hr. after "
6	1.33	0.204	0.261	0.128	2.03	96	43	1 " " "
11	5.00	0.222	0.294	0.134	2.19	98	45	2 hrs. " "
11	5.90	0.182	0.294	0.078	3.77	114	52	3 " " "
No urine.		0.222	0.294	0.150	1.96	114	50	4 " " "
17	5.90	0.209	0.234	0.186	1.25	114	52	5½ " " "
14	3.50	0.208	0.238	0.180	1.32	114	52	7 " " "
10	2.64	0.228	0.332	0.143	2.32	116	55	9 " " "
10	1.67	0.222	0.333	0.106	3.14	115	49	10 " " "
12	0.53	0.200	0.260	0.149	1.74	108	54	12½ " " "
19	Faint.	0.165	0.194	0.135	1.43	113	49	16 " " "
25	"	0.145	0.168	0.117	1.43	112	45	18½ " " "
170	0	0.129	0.138	0.119	1.16	111	47	23½ " " "
95	0	0.120	0.133	0.102	1.30	90	42	25 " " "
70	0	0.118	0.143	0.083	1.72	90	42	26½ " " "
250	0	0.119	0.122	0.100	1.07	80	39	31½ " " "
63	0	0.133	0.143	0.119	1.20	107	39	35½ " " "
50	0	0.113	0.125	0.091	1.37	97	35	38½ " " "
125	0	0.100	0.112	0.078	1.43	94	36	45⅜ " " "
40	0	0.080	0.098	0.053	1.84	96	40	50½ " " "

The totally depancreatized dog in Table XI had a noticeably high permeability of the corpuscles for sugar, both after the operation alone and after administration of 5 gm. of glucose per kilo by stomach. The ratios, nevertheless, varied with the usual unexplained irregularity, and there is no proof of any specific alteration due to pancreatectomy.

TABLE X.

Dog B2-79.

Severe diabetes. Fat feeding.

Date.	Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Remarks.
1915	*per cent*	*per cent*	*per cent*		
Oct. 7	Faint.	0.182	0.075	2.42	Blood before feeding 300 gm. lard.
	0	0.186	0.109	1.70	$4\frac{1}{4}$ hrs. after "
" 9	0	0.118	0.076	1.55	Blood before " 240 gm. suet.
	0	0.132	0.081	1.63	4 hrs. after "
" 11	0	0.136	0.081	1.68	Blood before " 360 gm. suet.
	0	0.166	0.112	1.48	$4\frac{3}{4}$ hrs. after "
" 14	0	0.137	0.125	1.09	Blood before " 500 gm. suet.
	0	0.128	0.083	1.54	4 hrs. after "
" 16	0	0.137	0.085	1.61	Blood before " 100 gm. suet.
	0	0.154	0.085	1.81	$5\frac{1}{2}$ hrs. after "
" 25	0	0.143	0.083	1.72	Blood before " 230 gm. suet.
	0	0.143	0.083	1.72	3 hrs. after "

Tables XII to XVIII show the effect of exercise in a series of dogs ranging from non-diabetic to severely diabetic. The negative effect upon the permeability of the corpuscles to sugar is in harmony with the results of experiments upon human diabetics.[7]

[7] Allen, Stillman, and Fitz,[4] Chapter V.

TABLE XI.

Dog C3-01.

Weight 15 kg. Total pancreatectomy.

Urine.								
Volume.	Glucose.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
cc.	*gm.*	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
	0	0.100	0.100	0.100	1.00	75	34	Blood before pancreatectomy.
		0.141	0.236	0.015 (?)	15.73	92	43	During ether anesthesia.
30	0.16	0.200	0.270	0.103	2.62	105	42	$3\frac{3}{4}$ hrs. after operation.
65	1.05	0.250	0.270	0.220	1.22	107	40	5 " " "
70	2.18	0.238	0.282	0.184	1.53	101	45	$7\frac{1}{2}$ " " "
120	4.30	0.263	0.365	0.138	2.64	95	45	$13\frac{1}{2}$ " " "
57	2.59	0.200	0.227	0.159	1.42	87	40	16 " " "
60	2.00	0.244	0.314	0.129	2.43	99	38	18 " " "
110	4.79	0.270	0.270	0.270	1.00	93	38	21 " " "
113	3.94	0.227	0.256	0.175	1.46	86	36	23 " " "
150	5.18	0.312	0.408	0.125	3.26	82	34	$26\frac{3}{4}$ " " "
100	5.00	0.334	0.500	0.063	7.93	81	38	$29\frac{3}{4}$ " " "
110	5.86	0.334	0.400	0.206	1.94	80	34	$32\frac{1}{2}$ " " "
115	5.00	0.355	0.400	0.268	1.49	75	34	$38\frac{1}{2}$ " " "
5 gm. glucose per kg. in 30 per cent solution by stomach tube, 48 hrs. after operation.								
90	4.27	0.244	0.358	0.050	7.10	83	37	Blood before feeding.
20	1.05	0.385	0.400	0.364	1.09	95	42	$\frac{1}{2}$ hr. after "
15	0.71	0.384	0.500	0.160	3.12	95	34	1 " " "
50	2.78	0.728	0.800	0.636	1.25	95	44	4 hrs. " "
65	0.16	0.666	0.910	0.367	2.47	80	45	8 " " "
No urine.		1.180	1.330	0.889	1.48	88	34	9 " " "
"	"	0.800	1.140	0.140	8.14	88	34	12 " " "

The dog in Table XVIII had not only severe diabetes and a high renal threshold, but also marked acidosis. It is thus noticeable that acidosis had no demonstrable influence upon the sugar concentration in the corpuscles.

TABLE XII.

Dog B2-00.

Depancreatized just short of diabetes. Exercise. July 28, 1915. Fasting.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0.097	0.105	0.079	1.32	80	31.0	Blood before exercise.
0.097	0.115	0.057	2.01	89	31.2	After 1 hr. of exercise.
0.094	0.102	0.079	1.29	70	35.0	" 4 hrs. " "

Oct. 5. Exercise with 3 gm. glucose per kg. by stomach tube.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.128	0.084	1.52	110	43.0	Blood before feeding and exercise.
	0.182	0.081	2.24	100	45.0	After ½ hr. of exercise and 40 min. after feeding.
0	0.134	0.100	1.34	94	38.0	After 1 hr. of exercise and 70 min. after feeding.
0	0.200	0.109	1.83	88	40.5	After 1 hr. of rest and 2 hrs. after feeding.

In Tables XIX to XXI, cold had no perceptible influence upon the sugar content of the corpuscles in non-diabetic, mildly diabetic, and severely diabetic animals.

Table XXII shows the effect of intravenous glucose injections in a small adult horse. Table XXIII illustrates starch feeding and administration of glucose subcutaneously and by stomach in a goat.

TABLE XIII.

Dog B2-01.

Depancreatized just short of diabetes. Exercise.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
			Fasting. July 28, 1915.			
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0.090	0.102	0.071	1.46	74	38.1	Blood before exercise.
0.114	0.143	0.058	3.46	78	34.2	After 1 hr. of exercise.
0.119	0.130	0.097	1.34	60	34.0	" 4 hrs. " "
			Fasting. July 29.			
0.102	0.110	0.085	1.29	80	31.8	Blood before exercise.
0.095	0.100	0.086	1.16	81	36.0	After 2 hrs. of exercise.
0.103	0.135	0.046	2.93	73	35.9	" resting 4 hrs.

4 gm. glucose per kg. by stomach tube. Oct. 7.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.078	0.105	0.046	2.28	113	46.0	Blood before feeding and exercise.
	0.114	0.140	0.086	1.63	120	48.0	After 5 min. of exercise.
	0.130	0.168	0.091	1.84	120	49.5	" 15 " " "
	0.100	0.125	0.073	1.71	119	48.0	" ½ hr. " "
Slight.	0.125	0.147	0.084	1.75	111		" 1 " " "
Doubtful.		0.148	0.111	1.33			" 1 " " rest.

TABLE XIV.

Dog B2-02.

Very mild diabetes. July 13, 1915. 3 gm. glucose per kg. in 30 per cent solution subcutaneously.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0.53	0.102	0.143	0.058	2.46	102	48.3	Blood after 1 hr. of exercise and before injection.
5.00	0.202	0.242	0.154	1.57	106	45.5	After 2 hrs. of exercise.
1.11	0.109	0.119	0.095	1.25	80	41.2	" 3 " " "
1.00	0.095	0.097	0.092	1.05	100	45.0	" 5 " " "
0.50	0.133	0.167	0.076	2.20	87	37.5	" 8 " " " Fed bread and soup.
0	0.107	9.108	0.105	1.03	100	40.0	After resting all night.
0	0.096	0.100	0.090	1.11	100	41.0	" 1 hr. of heavy exercise.

TABLE XV.

Dog B2-43.

Mild diabetes. June 29, 1915. Fed 100 gm. bread.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.118	0.125	0.109	1.14	103	43.0	Blood before feeding and exercise.
0	0.106	0.118	0.090	1.31	83	43.2	After $\frac{1}{2}$ hr. of hard exercise.
0	0.185	0.228	0.120	1.90	95	40.0	" $1\frac{3}{4}$ hrs. " rest.
0	0.091	0.091	0.091	1.00	98	40.5	" $2\frac{1}{2}$ " " exercise.

July 26 and 27. Fed 50 gm. solid glucose.

Plasma sugar / Corpuscle sugar		Remarks.
Without exercise.	With exercise.	
1.3		Before feeding 50 gm. glucose.
1.6	2.5	$1\frac{1}{4}$ hrs. after feeding.
1.4	1.4	$3\frac{1}{2}$ " " "
1.3	1.1	5 " " "

TABLE XVI.

Dog B2-63.

Mild diabetes. Bread feeding; Nov. 18, 1915, at rest; Nov. 19, with exercise.

Plasma sugar.		Corpuscle sugar.		Urine sugar.		Plasma sugar / Corpuscle sugar		Remarks.
Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19.	Nov. 18.	Nov. 19	
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
0.111	0.108	0.059	0.078	0	0	1.88	1.39	Before feeding. Exercise started.
0.127	0.095	0.087	0.087			1.46	1.09	½ hr. after feeding.
0.254	0.117	0.121	0.077	0	0	2.09	1.52	1 " " " Exercise stopped.
0.250	0.138	0.115	0.073	0.37	0	2.17	1.89	3 hrs. after feeding.
0.272	0.143	0.143	0.133	0.85	0	1.90	1.07	6 " " "

TABLE XVII.

Dog B2-79.

Severe diabetes. Comparison of days of exercise and rest, fasting.

Urine sugar.	Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.110	0.133	0.082	1.62	100	46.2	Before exercise July 17, 1915.
0	0.097	0.100	0.094	1.06	120	49.0	After 1 hr. of hard exercise.
0	0.102	0.109	0.093	1.17	105	45.3	Before exercise July 23.
0	0.093	0.105	0.080	1.31	125	49.5	After 3½ hrs. of exercise.
	0.115	0.169	0.061	2.77	110	50.0	Before exercise Sept. 25.
	0.128	0.143	0.114	1.25	110	52.0	After 1 hr. of exercise.

Feeding of 500 gm. beef lung; Nov. 8, at rest; Nov. 12, with exercise.

Plasma sugar / Corpuscle sugar		Remarks.
Nov. 8.	Nov. 12.	
1.2	1.6	Before feeding and exercise.
1.3	1.5	After 1 hr. of exercise.
1.4	1.1	" 2 hrs. " "
1.4	1.3	" 4 " " "

TABLE XVIII.

Dog B2-80.

Severe diabetes and acidosis. Exercise, fasting.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
Faint.	0.263	0.112	2.34	114	49.0	Blood before exercise July 23, 1915.
"	0.270	0.124	2.18	120	55.5	After 2½ hrs. of hard exercise.
0	0.208	0.124	1.68	118	50.5	Blood before exercise July 26.
0	0.208	0.129	1.61	125	53.6	After 1 hr. of hard exercise.
				Aug. 8.		
0.65	0.294	0.155	1.39	102	55.0	Before exercise.
0.55	0.294	0.127	2.31	101	56.0	After 10 min. of exercise.
1.67	0.322	0.204	1.58	94	61.0	" resting 1½ hrs.
1.23	0.333	0.140	2.38	105	64.5	" ½ hr. of exercise.
1.09	0.303	0.187	1.62	104	53.4	" 4 hrs. " resting.

TABLE XIX.

Dog B2-00.

Depancreatized just short of diabetes.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
				Aug. 6, 1916. Cold, fasting.		
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.110	0.106	1.04	85	32.9	Blood before going in ice box.
0	0.085	0.063	1.35	82	35.7	After 2 hrs. in ice box.
0	0.098	0.092	1.06	78	33.5	" 19 " " " " Fed 12 hrs. ago.
	0.107	0.089	1.20	74	34.7	" 24 " " " "
	0.125	0.046	2.72	70	30.7	" 2 " at room temperature.
	0.112	0.080	1.40	78		" 5½ " " " "
				Sept. 20, 1915. Cold and 3 gm. glucose per kg. by stomach tube.		
0	0.154	0.071	2.07	107	42	Before feeding and going into ice box.
	0.271	0.109	2.48	104	46	1 hr. after feeding.
0	0.295	0.125	2.36	100	51	2 hrs. " "
0	0.100	0.071	1.41	110	44	4 " " "
0	0.098	0.084	1.17	100	47	7 " " "
0	0.110	0.076	1.45	100	38	19 " " "

Tables XXIV and XXV show similar experiments in two sheep. The distribution of sugar between plasma and corpuscles in these species is seen to be not greatly different from that in the dog.

TABLE XX.

Dog B2-02.

Very mild diabetes. Cold and 3 gm. glucose per kg. by stomach tube.

Urine sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Hemoglobin.	Corpuscle volume.	Remarks.
per cent	*per cent*	*per cent*		*per cent*	*per cent*	
0	0.118	0.095	1.24	110	56.0	Before feeding and going in ice box.
Slight +.	0.228	0.115	1.98	104	54.0	1 hr. after feeding.
0	0.115	0.073	1.58	100	54.0	3 hrs. " "
0	0.100	0.065	1.54	100	52.2	6 " " "
	0.120	0.100	1.20	105	47.0	23 " " "

TABLE XXI.

Dog B2-79.

Severe diabetes. Feeding of 1 kg. beef lung. Nov. 23, 1915, in warm room, Nov. 30 in refrigerator.

Plasma sugar.		Corpuscle sugar.		Plasma sugar / Corpuscle sugar		Remarks.
Nov. 23.	Nov. 30.	Nov. 23.	Nov. 30.	Nov. 23.	Nov. 30.	
per cent	*per cent*	*per cent*	*per cent*			
0.133	0.250		0.200		1.25	Before feeding.
0.159	0.525	0.108	0.256	1.47	2.05	4 hrs. after feeding.
0.189	0.435	0.159	0.250	1.19	1.74	6 " " "

In the various preceding experiments, glucose and other foods were fed, and glucose also injected subcutaneously and intravenously, and no special differences of corpuscle permeability were found with the different modes of administration. Table XXVI shows the distribution of sugar between the plasma and corpuscles of sheep blood *in*

vitro under 3 conditions: (*a*) Glucose was added to the oxalated blood *in vitro*, for observing its passage into the corpuscles; (*b*) the corpuscles were washed with 0.85 per cent NaCl solution and then suspended in this solution with addition of glucose, in order to test the influence of the saline upon the permeability; (*c*) the oxalated blood was diluted

TABLE XXII.

Normal Pony.

Weight 350 kg.

Urine.		Blood sugar.	Plasma sugar.	Corpuscle sugar.	$\frac{\text{Plasma sugar}}{\text{Corpuscle sugar}}$	Corpuscle volume.	Remarks.
Volume.	Glucose.						
Injection of 2 gm. glucose per kg. in 50 per cent solution intravenously. Nov. 11, 1917.							
cc.	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	
	0	0.086	0.091	0.084	1.08	76	Blood before injection.
		0.825	1.250	0.305	4.10	45	5 min. after "
		0.446	0.675	0.258	2.61	55	15 " " "
432	5.40	0.391	0.568	0.287	1.98	63	½ hr. " "
13	6.45	0.204	0.286	0.176	1.62	75	1½ hrs. " "
			0.090	0.077	1.17		3½ " " "
		0.072	0.077	0.070	1.10	75	5 " " "
Same dose. Nov. 20.							
		0.115	0.125	0.112	1.09	78	Blood before injection.
		0.715	1.110	0.473	2.35	62	15 min. after "
1,240	5.05	0.500	0.625	0.446	1.70	70	½ hr. " "
2,725	4.74	0.192	0.279	0.172	1.62	82	1½ hrs. " "
		0.115	0.119	0.114	1.04	79	2½ " " "
640	2.15	0.064	0.056	0.066	0.08	80	4½ " " "

with the salt solution, so as to lower the concentration of sugar in the plasma and observe the passage of sugar out of the corpuscles. With reference to (*b*), the experiment of October 21 indicates that the salt solution altered the corpuscles in such a way as to cause them to contain less sugar. The result on November 14 was similar but less marked. The results under (*a*) and (*c*) showed the usual variability

TABLE XXIII.

Goat 1.

Normal.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Apr. 10, 1917.			
	per cent	*per cent*	*per cent*		*per cent*	
10.20 a.m.	0.058	0.074	0.040	1.85		Fed pan of oats.
5.00 p.m.	0.058	0.084	0.014	6.00	37.5	6 hrs. after feeding.
			Apr. 11.			
11.00 a.m.	0.047	0.068	0.012	5.66	37.7	12.30 p.m. Given subcutaneously 10 gm. glucose in 10 per cent solution.
1.30 p.m.	0.069	0.092	0.028	3.28	35.8	1 hr. after injection.
3.30 "	0.062	0.078	0.029	2.69	32.5	3 hrs. " "
6.00 "	0.056	0.069	0.033	2.09	36.0	
			Apr. 18.			
2.45 p.m.	0.062	0.098	0.024	4.08	49.1	Given by stomach tube 100 gm. glucose in 30 per cent solution.
3.45 "	0.079	0.098	0.046	2.13	36.9	
4.45 "	0.056	0.085	0.009	9.44	34.5	
6.00 "	0.063	0.088	0.016	5.50	35.0	
8.00 "	0.049	0.056	0.039	1.44	41.2	
			Apr. 23.			
11.30 a.m.	0.053	0.066	0.034	1.94		Given by stomach tube 300 gm. glucose in 10 per cent solution.
12.30 p.m.	0.097	0.137	0.069	1.98		
1.45 "	0.145	0.161	0.126	1.28	46.8	
3.30 "	0.119	0.152	0.071	2.13	41.0	Urine 98 cc. Sugar = 2.71 per cent.
4.30 "	0.130	0.133	0.126	1.05	42.3	
6.00 "	0.089	0.149	0.041	3.63	55.5	Urine 22 cc. Sugar = 6.05 per cent.

TABLE XXIV.

Sheep 1.

Normal.

Blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Sept. 18, 1917.		
per cent	*per cent*	*per cent*		*per cent*	
0.067	0.072	0.063	1.14	60	Blood before feeding oats and hay.
0.058	0.064	0.054	1.18	59	6 hrs. after feeding.
			Sept. 20.		
0.071	0.096	0.050	1.92	55	Blood before injecting 350 cc. 5 per cent glucose solution subcutaneously.
0.133	0.179	0.083	2.16	48	3 hrs. after injection.
0.125	0.154	0.093	1.66	48	$5\frac{3}{4}$ " " "
			Sept. 21.		
0.067	0.069	0.065	1.06	57	Blood before feeding 20 gm. glucose.
0.054	0.066	0.041	1.61	49	5 hrs. after feeding.
0.054	0.055	0.053	1.04	54	6 " " "
			Sept. 24.		
0.065	0.071	0.060	1.18	55	Blood before feeding 50 gm. glucose.
0.065	0.069	0.060	1.15	46	4 hrs. after feeding.
0.077	0.080	0.072	1.11	41	$5\frac{1}{2}$ " " "
			Oct. 21.		
0.067	0.078	0.054	1.44	46	Blood before injecting 20 gm. glucose subcutaneously.
0.161	0.250	0.099	2.52	59	$3\frac{1}{2}$ hrs. after injection.
0.053	0.072	0.016	4.50	34	$6\frac{1}{2}$ " " "
			Oct. 26.		
0.071	0.079	0.046	1.72		Blood before injecting 20 gm. glucose subcutaneously.
0.079	0.102	0.055	1.85		3 hrs. after injection.
0.059	0.076	0.049	1.55		$5\frac{1}{2}$ " " "
			Oct. 29.		
0.076	0.076	0.076	1.00		Blood before injecting 50 gm. glucose subcutaneously.
0.131	0.204	0.081	2.52		$3\frac{1}{2}$ hrs. after injection.
0.072	0.088	0.055	1.60		6 " " "

but on the whole resembled those *in vivo*, the corpuscle sugar concentration always running more or less below that of the plasma.

No attempt was made to determine the time required for passage of sugar into or out of the corpuscles *in vivo* or *in vitro*, but essentially the same ratios of distribution were found when the intervals between analyses were 5 to 30 minutes, as in Tables IX, XI, and XXII, or

TABLE XXV.

Sheep 2.

Normal.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.	Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Sept. 25, 1917.			
	per cent	*per cent*	*per cent*		*per cent*	
11.00 a.m.	0.054	0.062	0.043	1.44	43.3	Fed 50 gm. glucose.
12.00 m.	0.057	0.069	0.039	1.74	40.3	1 hr. after feeding.
2.00 p.m.	0.058	0.074	0.037	2.00	43.4	3 hrs. " "
4.00 "	0.058	0.056	0.060	0.93	49.3	5 " " " "
			Oct. 22.			
11.30 a.m.	0.051	0.100	0.037	2.70		Fed 20 gm. glucose.
4.30 p.m.	0.070	0.085	0.054	1.57	48.0	5 hrs. after feeding.
			Oct. 23.			
11.00 a.m.	0.079	0.112	0.031	3.61	41.1	Fed 100 gm. glucose.
3.00 p.m.	0.076	0.112	0.017	6.58	37.8	4 hrs. after feeding.
5.00 "	0.062	0.074	0.050	1.48	38.3	6 " " " "
			Dec. 28.			
9.00 a.m.	0.085	0.093	0.081	1.15	67.4	9.15 a.m. Given subcutaneously 43 gm. glucose in 12.5 per cent solution.
10.15 "	0.147	0.332	0.050	6.66	65.7	1 hr. after injection.
11.15 "	0.303	0.435	0.215	2.02	60.0	2 hrs. " "
12.15 p.m.	0.286	0.416	0.222	1.87	67.2	3 " " " "
2 15 "	0.218	0.370	0.094	4.15	55.2	5 " " " "

several hours as in other tables. The observations suggest that the distribution of sugar between plasma and corpuscles is governed not by permeability in any proper sense but by a coefficient of solubility between the two media.

TABLE XXVI.

Permeability of Sheep Corpuscles in Vitro.

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar		Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Analyzed.	Calculated.			
Sept. 18, 1918.							
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	
10.45 a.m.	0.067	0.072	0.065	0.064	1.1	60	To the remaining blood a pinch of glucose was added and allowed to stand at room temperature 1 hr.
11.45 "	0.880	0.880	0.880	0.880	1.0	55	
Oct. 21.							
10.30 a.m.	0.067	0.078	0.041	0.054	1.9	46	To the remaining blood 0.1 gm. glucose was added and blood allowed to stand at room temperature 2 hrs.
12.30 p.m.	1.140	1.510	0.667	0.325	2.3	44	
12.30 "	0.910	1.510	0.286	0.098	5.2	43	Corpuscles of the 10.30 a.m. blood centrifuged, washed with salt solution, then made to 10 cc. with salt solution; 0.1 gm. glucose added and allowed to stand at room temperature 2 hrs.
2.00 "	0.161	0.250	0.084	0.098	3.0	59	Blood taken 4½ hrs. after subcutaneous injection of 50 gm. glucose. Remaining blood diluted ⅓ with salt solution and allowed to stand 1 hr.
3.00 "	0.082	0.132	0.062	0.056	2.1	33	

TABLE XXVI—*Concluded.*

Time.	Whole blood sugar.	Plasma sugar.	Corpuscle sugar.		Plasma sugar / Corpuscle sugar	Corpuscle volume.	Remarks.
			Analyzed.	Calculated.			
				Nov. 14.			
	per cent	*per cent*	*per cent*	*per cent*		*per cent*	
11.30 a.m.	0.065	0.132	0.065	0.056	2.0	53	To the remaining blood 0.1 gm. glucose added and allowed to stand 2 hrs. at room temperature.
1.30 p.m.	2.00	2.78	1.35	1.41	2.1	57	
1.30 "	2.00	2.78	0.835	1.05	3.3	34	Corpuscles of 11.30 a.m. centrifuged and washed with salt solution, made to 10 cc. with salt solution, and 0.1 gm. glucose added.
3.00 "	0.169	0.217	0.062	0.122	3.5	51	3 hrs. after subcutaneous injection of 50 gm. glucose.
3.30 "	0.077	0.149	0.055		2.7	31	Remaining blood diluted $\frac{1}{3}$ with salt solution and allowed to stand $\frac{1}{2}$ hr.
4.00 "	0.114	0.118	0.056	0.106	2.1	34	Blood diluted $\frac{1}{3}$ with salt solution after standing 1 hr.

CONCLUSIONS.

1. No specific alteration of the distribution of sugar between the blood plasma and corpuscles was found in experiments with different quantities and modes of administration of glucose, different degrees of pancreatectomy and diabetes, lipemia, acidosis, exercise, cold, or different levels of the renal threshold.

2. The concentration of sugar in the corpuscles is normally a little below that in the plasma. The discrepancy in favor of the plasma generally becomes greater as the blood sugar rises. These relations hold good in the several animal species examined, and in experiments *in vivo* and *in vitro*. The statement that in the declining stage of hyperglycemia the corpuscles retain sugar longer than the plasma was not confirmed. The concentration in the corpuscles rarely equals that

in the plasma, and the finding of an excess in the corpuscles is probably to be interpreted as an analytical error.

3. The term permeability as commonly used in relation to the sugar content of the corpuscles carries the idea that this is governed by a partially permeable membrane surrounding the corpuscle, and further that the solubility of glucose in the corpuscle substance is the same as in the plasma. This conception of permeability has received support from the assertions that the corpuscles take up and give off sugar more slowly than the plasma. It may possibly derive some support from the fact that washing in physiological salt solution changes the corpuscles in such a way that they take up less sugar, as reported by former authors. In view of the almost uniformly lower concentration in the corpuscles, however, and the quickness of adjustment on this basis whether the blood sugar rises or falls, it seems a more probable assumption that the coefficient of distribution depends upon solubility, glucose being more freely soluble in the plasma than in the corpuscle substance, perhaps because of the lipoid content of the latter.

4. Inasmuch as the sugar content of the corpuscles is subject to considerable irregularities from unknown causes and without known physiological significance, plasma analyses should be preferred to those of whole blood for experimental and clinical purposes.

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BIOLOGICAL STUDY OF THE HEMOPHILIC BACILLI.

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In 1892 Pfeiffer[1] described a small Gram-negative bacillus which he associated with the disease influenza. Ultimately this bacillus was quite widely considered as the cause of influenza. The bacillus of Pfeiffer, or *B. influenzæ*, as it was eventually named, is a very small, non-motile, non-sporulating, faintly staining organism with rounded ends. It is irregular in form with a tendency to show bipolar staining. Coccoid forms are also frequently seen, and occasionally small chains of bacilli occur. Pfeiffer recovered the bacillus by smearing pus from bronchial secretions over serum agar, but subcultures failed to grow. He finally discovered that it was the hemoglobin in the pus which enabled the bacillus to grow. Thus this organism came to be known as a hemophilic bacillus. Since Pfeiffer's discovery, various Gram-negative hemophilic bacilli have been described, such as the pseudoinfluenza bacillus, Jochmann's bacillus, Muller's "trachoma bacillus," the Koch-Weeks bacillus, etc. The more recent studies of Wollstein[2] would seem to indicate that while there are minor morphological and cultural differences between these hemophilic bacilli, the distinctions are so slight that the various hemophilic bacilli should be considered identical with *B. influenzæ* or, at most, as varieties of the same species.

The epidemic of influenza in 1918 called to attention the lack of knowledge concerning the biology and epidemiology of the hemophilic bacilli despite the large amount of work done in connection with influenza. In a study of the occurrence of *B. influenzæ* in normal mouths, Pritchett and Stillman[3] described a hemophilic bacillus strikingly similar to, but distinguished from *B. influenzæ* by its ability to hemolyze blood. The colony of this organism cannot be differentiated from that of *B. influenzæ* on oleate agar or chocolate medium, and morphologically the differences are so slight that they cannot be relied upon. As a rule, the so called Bacillus X is slightly larger and coarser than *B. influenzæ* in stained films. The easiest method of differentiation is by growth on blood agar, upon which Bacillus X shows varying degrees of hemolysis. The majority of the strains of this organism actively hemolyze the surface of a blood agar plate and also hemolyze blood broth. An occasional strain is encountered, however,

[1] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1892, xiii, 357.

[2] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

[3] Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

whose hemolytic powers are not well developed. If good growth is obtained in plain broth enriched with 2 per cent blood extract, hemolysis may be demonstrated by the use of a 5 per cent solution of washed rabbit blood corpuscles. 1.5 cc. of an 18 hour broth culture added to 0.5 cc. of the washed blood cells and placed in a water bath at 37.5°C. for 1 hour usually cause complete hemolysis. Jordan[4] in 1919 called attention to the fact that certain strains of *B. influenzæ* produced indole. Wadsworth and Wheeler,[5] in their work with *B. influenzæ*, note the production of gas, and also the fermentation of monosaccharides by some strains. Rivers[6] reported that certain strains of *B. influenzæ* produced indole and amylase and could reduce nitrates to nitrites.

In 1906 Bordet and Gengou[7] succeeded in cultivating a small, ovoid, Gram-negative bacillus which they had observed in the sputum of children suffering from pertussis. This bacillus, although very similar morphologically to *B. influenzæ*, is less pleomorphic, slightly larger, and generally appears more ovoid. After frequent subcultures *B. pertussis* grows on ordinary media without the presence of hemoglobin. It grows much more slowly during the first 24 hours of incubation than *B. influenzæ* On blood agar a fine film of growth is barely visible at the end of 24 hours, while at the end of 48 hours a heavy grayish growth has developed which is very different from the appearance of *B. influenzæ* grown under the same conditions. Ferry and Noble[8] have stated that there is an apparent close relation between *B. pertussis* and *B. bronchisepticus*, although the latter grows luxuriantly on ordinary media and is motile. The bacillus of rabbit septicemia, while not hemophilic, presents a striking morphological likeness to *B. influenzæ*. Because of the morphological resemblance of these various bacilli to *B. influenzæ* a few strains are included for comparison in the present study of the hemophilic bacilli.

As the hemophilic bacilli are delicate organisms which do not grow readily on artificial media special attention must be paid to minute details of technique. This fact is well exemplified by the difficulty with which *Bacillus influenzæ* was cultivated before the use of special media such as oleate agar and chocolate agar and probably in large part accounts for our lack of knowledge of the biology of this delicate organism. Freshly prepared medium adjusted to the optimum hydrogen ion concentration, pH 7.3 to 7.5, is essential for growth.

[4] Jordan, E. O., *J. Am. Med. Assn.*, 1919, lxxii, 1542.

[5] Wadsworth and Wheeler, in Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 457.

[6] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 50.

[7] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1906, xx, 731.

[8] Ferry, N. S., and Noble, A., *J. Bact.*, 1918, iii, 193.

EXPERIMENTAL.

In the present paper are reported the data obtained during an investigation of the hemophilic bacilli recovered from the throats and sputum of patients suffering from acute influenza and lobar pneumonia, and from the throats and saliva of healthy individuals. The study includes the facts obtained concerning (1) final hydrogen ion concentration, (2) sugar fermentation, (3) indole production, (4) nitrate reduction, and (5) gas production. In addition to the strictly hemophilic organisms, *Bacillus influenzæ* and the so called Bacillus X, described by Pritchett and Stillman, a few strains of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* have been included for comparative study.

Upon isolation the majority of the strains of *Bacillus influenzæ* and all the strains of Bacillus X were plated on dextrose agar to which no hemoglobin had been added. In no instance was growth obtained in the hemoglobin-free medium. After prolonged artificial cultivation, in some instances over 2 years, all these strains were again plated on ascitic dextrose agar without hemoglobin. They invariably failed to grow on media which lacked hemoglobin. All media used in this study were enriched by the addition of 4 per cent defibrinated rabbit blood or 2 per cent blood extract. The latter was substituted for defibrinated rabbit blood in the case of the hemolytic Bacillus X, since the hemolysis produced by this organism might mask certain reactions. In many instances also in the work with the non-hemolytic hemophilic bacilli (*Bacillus influenzæ*) when defibrinated blood might interfere with the determination of a reaction, blood extract was used to enrich the media. The extract was made as advised by Wollstein.[9] Defibrinated rabbit blood was boiled for 2 minutes. The clot was finely broken and centrifuged. The resulting extract was added to the media in such a proportion as to give about 2 per cent enrichment. Since Winchell and Stillman[10] found that the optimum hydrogen ion concentration for growth of *Bacillus influenzæ* is between pH 7.3 and 7.5, all media used in the present study had an initial reaction of about pH 7.4 unless otherwise stated.

[9] Wollstein, M., *J. Exp. Med.*, 1919, xxx, 555.

[10] Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.

Non-Hemolytic Hemophilic Bacilli (Bacillus influenzæ).

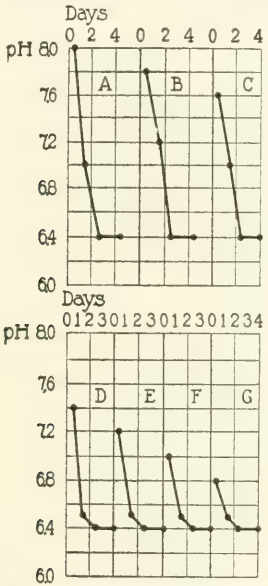
Hydrogen Ion Concentration.—Since the final hydrogen ion concentration reached by growth of an organism in a given medium is a biological constant of differential value, this reaction was determined n the study of the hemophilic bacilli. The colorimetric method, with phenol red and brom cresol purple as indicators, was used to determine the hydrogen ion concentration, and the readings were confirmed by the electrometric method in a number of experiments.

In the usual dextrose meat infusion broth containing 0.5 per cent sodium chloride, *Bacillus influenzæ* attains a final acid reaction of about pH 6.2. If 0.2 per cent sodium phosphate is substituted for the 0.5 per cent sodium chloride in this medium, as is customary for routine purposes in these laboratories, the buffer value is so great that the changes in reaction are insignificant. Since *Bacillus influenzæ* evidently produces relatively small amounts of acid it is desirable in determining the final hydrogen ion concentration to use the medium containing less buffer. Consequently, throughout this work broth containing 0.5 per cent sodium chloride has been used.

In order to determine whether the initial hydrogen ion concentration of the medium had any effect on the final reaction, separate portions of dextrose broth, adjusted to varying hydrogen ion concentrations from pH 8 to 6.8, were inoculated with the same culture of *Bacillus influenzæ*. Text-fig. 1 shows the initial hydrogen ion concentration of the broth when inoculated and the hydrogen ion concentration of the cultures after 20, 44, and 70 hours incubation. From this it is seen that the initial reaction bears no relation to the final hydrogen ion concentration, which is pH 6.4 in each instance.

The relation of oxygen supply to growth was next tested by inoculating a series of 100 cc. Erlenmeyer flasks containing 25 cc. of dextrose broth and a set of large test-tubes containing a similar amount of broth. The initial hydrogen ion concentration of the media was pH 7.3. The test-tube cultures were incubated in an upright position. Colorimetric readings of the hydrogen ion concentrations of the cultures were made after 1, 3, 7, and 14 days incubation. After 24 hours incubation the flask cultures had attained a pH of 6.4, while the test-tube cultures did not reach this end-point until the 7th to 10th day.

It was noted that the macroscopic appearance of the cultures is not a criterion of the hydrogen ion concentration. Cultures which are very turbid and apparently have grown well, when tested may be found not to have reached their lower limit of acid production.



TEXT-FIG. 1. Effect of different initial hydrogen ion concentrations on final hydrogen ion concentrations of *B. influenzæ*.

The final pH was determined on a large series of cultures of *Bacillus influenzæ*. It was found to lie between pH 6 and 6.4. The length of incubation necessary before the different strains, and even the same strain, reach their final hydrogen ion concentration varies. Some strains reach the final reaction at the end of 24 hours; others, at times, do not reach pH 7 even after 14 days incubation in large slanted test-tubes. This variability of growth of *Bacillus influenzæ* has been

encountered throughout the present study. In working with this organism experiments giving negative results must be repeated, since the results may be due merely to insufficient growth.

Sugar Fermentation.—Since all the cultures of *Bacillus influenzæ* ultimately reached a final hydrogen ion concentration of at least pH 6.4 in dextrose broth, a reaction sufficiently acid to be detected by the Andrade indicator, the ability of these organisms to ferment different sugars was tested. It was found that sugar-free broth could not be used as nutrient substrate even after enrichment with blood extract and the addition of the test substance, for *Bacillus influenzæ* did not readily produce acid in this medium. Since meat infusion broth contains varying amounts of muscle sugar which might possibly modify fermentation reactions, Dunham's peptone solution was employed as a basis for the sugars, which were added in 1 per cent concentration. The peptone solution does not contain sufficient reducing sugar to give a positive test with Benedict's reagent. In Dunham's peptone solution enriched with 2 per cent blood extract *Bacillus influenzæ* grows luxuriantly. All culture tubes were incubated in a slanted position so as to expose to the air as large a surface of the medium as possible, since it has been shown that acid production is more rapid under these conditions.

The results of the sugar fermentation are given in Table I. It is seen that almost all strains of *Bacillus influenzæ* produce acid in the monosaccharides, dextrose and galactose. Acid production is less marked and more irregular with levulose. Some strains of *Bacillus influenzæ* fermented the polysaccharides, maltose and saccharose, and to a less extent dextrin. No strains could attack mannitol or lactose. A number of strains were tested against inulin, but the results were so consistently negative that this test substance was subsequently discarded. It is evident that the sugar reactions are irregular. This irregularity is especially noticeable if the same strain is repeatedly inoculated in the same sugar. Although good growth was apparently present, a culture which had been repeatedly positive in dextrose, for instance, at times failed to produce sufficient acid to cause the Andrade indicator to change color. The same factors which sometimes prevented cultures from reaching their final hydrogen ion concentration of pH 6.4 in dextrose broth apparently were acting here.

TABLE I.

Source, Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Non-Hemolytic Hemophilic Bacilli (B. influenzæ).

Source of strain.	Total No. of strains	Strains fermenting sugars.																Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Acute influenza	21	20	95	20	95	13	61.9	4	19	2	9	3	14	0	0	0	0	14	66	21	100	1	4	0	0
Convalescents from influenza	11	11	100	9	82	10	91	0	0	0	0	1	9	0	0	0	0	8	72	11	100	0		0	0
Lobar pneumonia	18	18	100	18	100	8	44	2	11	1	5	1	5	0	0	0	0	12	66	18	100	1	5	0	0
Normal individuals during winter of 1918–19	33	32	97	28	85	24	72	5	15	6	18	4	12	0	0	0	0	18	54	31	94	2	6	0	0
Normal individuals during Sept., 1919	11	10	91	9	82	8	72	5	45	5	45	5	45	0	0	0	0	5	45	11	100	5	45	0	0
Normal individuals during Feb., 1920	25	25	100	24	96	24	96	15	60	16	64	14	56	0	0	0	0	6	24	25	100	7	28	0	0
Total	119	116	97	108	90	87	73	31	26	30	25	28	23	0	0	0	0	63	53	117	98	16	13	0	0

Hydrogen ion determinations were made on a number of strains grown in media containing different sugars. The strains of *Bacillus influenzæ* which showed fermentation with the Andrade indicator had a final hydrogen ion concentration of pH 6 to 6.4 which corresponded to that obtained in dextrose broth when no Andrade indicator was present. An interesting result was the reaction of *Bacillus influenzæ* in lactose. In this sugar there was a definite increase in alkalinity, as illustrated by Table II. If plain peptone solution, peptone solution plus dextrose, and peptone solution plus lactose are inoculated with the same culture of *Bacillus influenzæ* and incubated for 10 days the dextrose culture becomes acid, but the culture in plain peptone and that containing lactose become alkaline. Bacillus X produces a similar increase in alkalinity in lactose media.

TABLE II.

Final Hydrogen Ion Concentration of the Non-Hemolytic Hemophilic Bacilli in Peptone Solution, Peptone Solution Plus Lactose, and Peptone Solution Plus Dextrose.

Solution.	pH
Peptone	8.4 +
" + 1 per cent lactose	8.4 +
" + 1 " " dextrose	6.0

In the course of the work on sugar fermentation Bronfenbrenner's double indicator "CR" was tested.[11] This indicator is composed of equal parts of a 0.5 per cent aqueous solution of China blue and a 1 per cent alcoholic solution of rosolic acid. China blue appears blue or bluish green in the presence of acid, and colorless in the presence of alkali. Rosolic acid, which is colorless in an acid medium, becomes pink in an alkaline medium.

Dunham's peptone solution containing 1 per cent sugar concentrations and enriched with 2 per cent blood extract was used. 1 per cent CR indicator was substituted for the Andrade indicator previously used. Only two sugars, dextrose and lactose, were tested. Representative strains of the Gram-negative bacilli under observation were used in determining the value of CR as an indicator of acid and alkali production in the presence of these two sugars.

[11] Bronfenbrenner, J., *J. Med. Research*, 1918–19, xxxix, 25.

The hemolytic and non-hemolytic strains of the strictly hemophilic bacilli produced acid in the dextrose CR medium and alkali in the lactose CR medium as indicated by the definite color changes after 48 hours incubation. The intensity of the reaction increases with prolonged incubation. The bacillus of rabbit septicemia also produced acid in the presence of dextrose and alkali in the presence of lactose. The strains of *Bacillus pertussis* and *Bacillus bronchisepticus* produced alkali in both the dextrose and lactose media. These results correspond exactly with the results obtained with similar sugar media in which Andrade indicator replaced CR, as will be seen by referring to Tables I, III, and IV.

CR, in the concentration used, does not appear to be bactericidal for any of the strains tested in the fluid medium described, for good growth was obtained in each instance and the color changes which occurred were striking. In poured oleate hemoglobin agar plates to which CR and the desired sugar had been added in 1 per cent concentration, striking color changes did not occur. In the concentration used in solid medium CR did not appear to be of value as a differential indicator.

Indole Production.—Jordan has called attention to the production of indole by *Bacillus influenzæ*. The indole production by cultures included in this study was tested by Ehrlich's para-dimethylaminobenzaldehyde method. Of the 119 strains of *Bacillus influenzæ* studied, 63, or 53 per cent, produced indole. It was found that indole was present at times after only 18 hours incubation at 37°C. and was produced for as long a period as 3 weeks. Indole was produced quite regularly in plain blood broth cultures, but slightly more positive reactions were obtained if Dunham's peptone solution enriched with blood extract was used. This may be due to the fact that the defibrinated blood masked delicate reactions. The same irregularity of reaction that was noted in the fermentation of sugar by *Bacillus influenzæ* was observed in the production of indole; occasionally an indole-producing strain, which apparently had grown luxuriantly, failed to produce indole. Hence the necessity of repeated tests before a culture may be definitely classified as a non-indole producer. A possible relation exists between indole production and sugar fermentation. Only one indole-producing strain fermented the polysaccharides.

Nitrate Reduction.—Of the 119 strains of *Bacillus influenzæ* studied, 117 were able to reduce nitrates to nitrites. This reduction as a rule occurred after 24 hours incubation. Like other reactions with these delicate organisms, at times negative results were obtained without apparent reason. In a few instances a culture which had given a positive nitrite reaction after 24 hours incubation failed to give it after 10 days incubation.

Gas Production.—The ability of the hemophilic bacilli to produce gas was first tested by making stab cultures in 1 per cent dextrose agar to which a small amount of blood extract had been added. Under these partial anaerobic conditions luxurious growth was obtained with all strains. Of the 119 non-hemolytic strains, sixteen, or 13 per cent, were found to produce gas. The gas appeared usually after 48 to 72 hours incubation, but with several strains did not appear until later. The time of incubation necessary before the appearance of gas varied on different occasions when the same strain was used. The amount of gas produced was never very great.

Stab cultures were made with blood extract dextrose medium containing agar in concentrations of 1, 1.5, and 2 per cent. 1 per cent agar seemed to be the most favorable concentration for the demonstration of gas. Gas production was demonstrated also in shake cultures of dextrose blood extract agar. With the exception of one strain, the non-hemolytic gas-producing organisms did not produce gas in Smith fermentation tubes when meat infusion broth or Dunham's peptone solution containing dextrose and blood extract was used. It appears, therefore, that a solid medium is more suitable than a fluid medium for the production of gas by these non-hemolytic strains of hemophilic bacilli.

Hemolytic Hemophilic Bacilli (Bacillus X).

The hemolytic hemophilic bacilli reach a final hydrogen ion concentration which varies from pH 6.4 to 5.8. Most strains ferment dextrose, maltose, and saccharose readily and quite regularly, and utilize galactose, levulose, and dextrin less easily and more irregularly. The same irregularities and difficulties of growth have been encountered with this organism as with the non-hemolytic type, although it is not quite so variable. Of the twenty-nine hemolytic strains studied only

three produced indole after repeated attempts. All the strains reduced nitrates to nitrites. Only four strains, or 13 per cent, showed the production of gas in dextrose blood extract agar. With these organisms the gas appeared usually after 24 to 48 hours of incubation. With only one of the strains could gas production be demonstrated in a Smith tube with meat infusion broth containing dextrose and blood extract. Stab and shake cultures were made in a similar manner as described above in connection with the non-hemolytic organisms.

TABLE III.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Hemolytic Hemophilic Bacilli (Bacillus X) Isolated from Normal Individuals during the Winter of 1919–20.

Total No. of strains.	Strains fermenting sugars.																Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
	Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
29	29	100	3	10	9	31	24	82	23	79	15	51	0	0	0	0	0	0	3	10	29	100	4	13	29	100

Relation between Indole Formation and Gas Production.

An apparent relation between indole formation and gas production can be observed. All the non-hemolytic gas-producing strains are non-indole producers and comprise strains that ferment mono- as well as polysaccharides. The hemolytic gas-producing strains, with one exception, produce indole. These hemolytic gas-producing strains do not ferment sugars so readily as the other organisms in this group.

Comparative Study of Bacillus pertussis, the Bacillus of Rabbit Septicemia, and Bacillus bronchisepticus.

Table IV shows the results of a comparative study of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* in connection with the hemophilic bacilli.

TABLE IV.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of Strains of B. pertussis, the Rabbit Septicemia Bacillus, and B. bronchisepticus.

Bacillus.	Total No. of strains.	Strains fermenting sugars.																				Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.											
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.			No.	Per cent.	No.	Per cent.	No.	Per cent.	No	Per cent.
B. pertussis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0
Rabbit septicemia bacillus	4	4	100	4	100	4	100	0	0	4	100	0	0	4	100	0	0	0	0			4	100	2	50	0	0	0	0
B. bronchisepticus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			0	0	0	0	0	0	0	0

Bacillus pertussis.—The four strains of *Bacillus pertussis* studied were stock cultures which had been under artificial cultivation for a considerable length of time. They had a final hydrogen ion concentration of pH 8 to 8.6 in dextrose broth and failed to produce acid in any of the sugars tested. Neither did they produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

Bacillus of Rabbit Septicemia.—The four strains of the bacillus of rabbit septicemia had a final hydrogen ion concentration of pH 6 and produced acid in dextrose, galactose, levulose, saccharose, and mannitol. They did not produce acid in maltose, lactose, dextrin, or inulin. These organisms produced indole slowly, and two strains, or 50 per cent, reduced nitrates to nitrites. In dextrose agar stab cultures there was good growth in the stab, but no gas was produced.

Bacillus bronchisepticus.—The two strains of *Bacillus bronchisepticus* studied had a final hydrogen ion concentration of pH 9.2 in dextrose broth and failed to produce acid in any of the sugars tested. They did not produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

DISCUSSION.

The small Gram-negative hemophilic bacilli which have gradually come to be considered as belonging to one group of organisms and to which the name *Bacillus influenzæ* has been given, appear in the light of the present study to be rather a group of closely allied bacilli which have demonstrable biological differences. The bacillus which Pfeiffer first described and associated with clinical influenza is now questioned as being the etiological factor in the spread of this disease. However, the percentage of cases in which the bacillus of Pfeiffer has been recovered is great enough to indicate that this organism may be at least a secondary invader. Since the first description of this hemophilic bacillus in 1892 by Pfeiffer, little has been added to our knowledge of its biological characteristics.

In this study we have found that the hemophilic bacilli observed divide themselves naturally into two large groups according to their

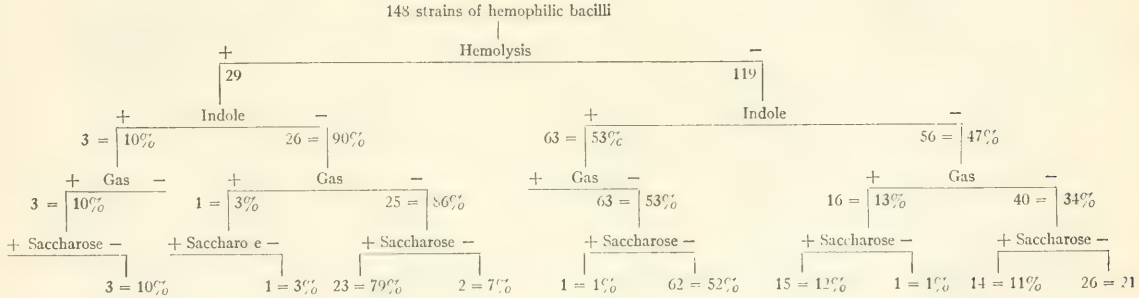
ability to hemolyze whole blood. The hemolytic group comprises the organisms originally described as Bacillus X by Pritchett and Stillman, and occurs in normal mouths. Many of these hemolytic bacilli have no doubt been confused with the non-hemolytic variety due to the almost universal use of chocolate medium. On oleate agar the colonies are so similar that they cannot be distinguished, and morphological differences are so slight as not to be reliable. Organisms of the hemolytic type (Bacillus X) do not live so long in culture media as those of the non-hemolytic type. They are best preserved at a low temperature. A few strains have been found to live from 2 to 3 weeks if kept in blood broth in the ice chest, but in order to be successfully preserved in stock cultures they must be transplanted every 6 or 7 days. At room temperature Bacillus X survives about 5 days, while at 37.5°C. it remains viable about 10 days. The non-hemolytic group (*Bacillus influenzæ*), on the other hand, remains viable for a month or more at room temperature in blood broth.

The hemolysin produced by the hemolytic type is quite stable, retaining its activity after being kept on ice for 6 weeks to several months. It can be demonstrated in a young broth culture after 2 hours incubation at 37°C. It is non-filterable and is destroyed by heating for $\frac{1}{2}$ hour at 56°C. Different strains vary, however, in their ability to produce hemolysis. The hemolytic bacilli are non-pathogenic for rabbits, guinea pigs, and mice.

Both the non-hemolytic and hemolytic groups of hemophilic bacilli attain a final hydrogen ion concentration of approximately pH 6.4, although the hemolytic group may reach pH 5.8. Both produce acid in dextrose, but in both groups only certain strains ferment saccharose. The greater ability of the hemolytic organisms to ferment sugars may be a basis for further differentiation.

A tentative classification, graphically illustrated below, defines a small subgroup of the hemolytic group formed by the strains which produce indole and gas but do not ferment saccharose. These strains appear to ferment sugars less readily and require further study to determine whether the indole-producing strains are also gas producers. The greater number of hemolytic strains, however, do not produce indole or gas, but ferment saccharose.

Tentative Classification of the Hemophilic Bacilli Based on Hemolysis, Indole and Gas Production, and the Fermentation of Saccharose.



The non-hemolytic organisms are subdivided into two fairly even groups comprising indole-producing and non-indole-producing strains. None of the indole producers forms gas, in contrast with the hemolytic group. With one exception, none of the non-hemolytic indole-producing strains ferments saccharose. A large majority of the non-indole-producing organisms of the non-hemolytic type do not form gas and do not ferment saccharose. With a single exception, all the indole-negative strains which form gas also ferment saccharose.

One of the most striking features of this classification may be best illustrated by Table V, which represents a comparison of three factors differentiating the hemolytic and non-hemolytic groups of the hemophilic bacilli. Here it is seen that the majority of the strains of the hemolytic type do not produce indole or gas, but ferment saccharose,

TABLE V.

Three Differential Factors of the Hemolytic and Non-Hemolytic Groups of the Hemophilic Bacilli.

Differential factors.	Hemolytic group.		Non-hemolytic group.	
	Positive.	Negative.	Positive.	Negative.
	per cent	*per cent*	*per cent*	*per cent*
Saccharose	79	21	25	75
Gas	13	86	13	87
Indole	10	90	53	47

while the reverse is true of the non-hemolytic type; that is, the majority of the non-hemolytic organisms do not produce gas but also do not ferment saccharose. It will be noted that the non-indole and indole-producing strains of the non-hemolytic type fall into almost even groups.

The classification made in this study is merely a tentative one. Undoubtedly when the technique of the reactions is more nearly perfected and a larger number of hemophilic bacilli has been studied, the group differentiations will be more striking and regular.

Although the number of strains of *Bacillus influenzæ* employed is too small to warrant any definite conclusions, it would seem that the non-hemolytic bacilli isolated from individuals suffering with and recovering from respiratory infections and those isolated from normal mouths during the epidemic period differ biologically in certain re-

spects from the strains recovered from normal individuals during the winter of 1919–20. This point is illustrated by Table VI. It is seen that the group of non-hemolytic hemophilic bacilli recovered from normal mouths during the winter of 1919–20 shows a higher percentage of strains which ferment the polysaccharides, maltose, saccharose, and dextrin, and more strains which produce gas, but fewer indole-producing strains.

TABLE VI.

Comparison of the Strains of Non-Hemolytic Hemophilic Bacilli Recovered from Respiratory Infections and Normal Mouths during Epidemic Period of 1918 and Strains Recovered from Normal Individuals during the Winter of 1919–20.

Source of strain.	Total No. of strains.	Strains fermenting sugars.														Strains producing indole.		Strains producing gas.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose		Dextrin.							
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.			No.	Per cent.	No.	Per cent.
Respiratory diseases and normal mouths during epidemic of 1918, and respiratory diseases during 1919–20.	83	81	97	75	90	55	66	11	13	9	10	9	10			52	62	4	4
Normal mouths during winter of 1919–20.	36	35	97	33	91	32	88	20	55	21	58	19	52			11	30	12	33

The Gram-negative bacilli which are not hemophilic and which have been studied because of their morphological similarity can be easily differentiated from the hemolytic and non-hemolytic hemophilic bacilli of the influenza type. The bacillus of rabbit septicemia shows a striking similarity to members of the non-hemolytic hemophilic group in the limiting hydrogen ion concentration, indole production, and nitrate reduction. On the contrary, *Bacillus pertussis* and *Bacillus bronchisepticus*, while resembling each other in certain reactions, do not simulate the strictly hemophilic group. These organisms have a markedly alkaline final hydrogen ion concentration, and do not produce indole or reduce nitrates.

CONCLUSIONS.

1. The hemophilic bacilli can be divided into two large groups according to the ability of certain strains to produce hemolysis.

2. Both the hemolytic and the non-hemolytic groups may be further subdivided according to the ability of some strains to produce indole, to form gas, and to ferment certain carbohydrates.

3. The hemophilic bacilli of both the hemolytic and the non-hemolytic varieties when grown in meat infusion broth containing 1 per cent of dextrose reach a final hydrogen ion concentration of about pH 6.4. In addition, practically all the strains possess the power to reduce nitrates to nitrites.

We wish to express our thanks to Dr. N. S. Ferry for a culture of *Bacillus bronchisepticus*, to Dr. Martha Wollstein for cultures of *Bacillus pertussis* and the bacillus of rabbit septicemia, to Dr. Anna Williams for cultures of *Bacillus pertussis*, to Dr. F. S. Jones for a culture of *Bacillus bronchisepticus*, and to the American Museum of Natural History for a culture of *Bacillus pertussis*.

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EXPERIMENTAL STUDIES IN DIABETES.

Series II. The Internal Pancreatic Function in Relation to Body Mass and Metabolism.*

5. The Influence of Fever and Intoxication.

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(Received for publication August 10, 1920.)

The accuracy with which the metabolism of cold-blooded animals can be regulated through the temperature was one of the reasons for the attempt to produce diabetes in them at the outset of this investigation. When the production of a satisfactory type of diabetes proved impossible, the research was thrown back entirely upon mammalian experiments, where the disturbing factors are greater. Some observations were made concerning the effects of fever and of external cold.

No discussion of the literature will be undertaken, beyond reference to a review (1) of the earlier literature, and a more recent paper by Freund and Marchand (2), which show that elevation of body temperature is generally accompanied by elevation of blood sugar, but terminal collapse may be accompanied by hypoglycemia; the rise of body temperature in itself tends to increase sugar tolerance, and lowered tolerance or glycosuria are attributable to intoxication or sometimes to pancreatic damage. Infections are known to be one of the worst agencies in aggravating human diabetes. The effect of aseptic elevation of temperature seems not to have been tested. It was desired in the present research to compare several forms of infectious and non-infectious fever in their influence upon partially depancreatized dogs. The only results actually permitted by circumstances

* The first four papers of this series are published in the American Journal of the Medical Sciences.

consisted in records of a number of animals which acquired chance infections, and one research concerning the gas bacillus. The observations will be classified according to the site of the infections.

Distemper. For this purpose canine distemper is closely comparable to human tuberculosis. Diabetes plainly increases the susceptibility of dogs and still more of puppies to this infection, in the sense that they both acquire it and succumb to it more easily. Tuberculosis seriously lowers the food tolerance and increases the tendency to glycosuria and hyperglycemia in human patients even in the earlier non-febrile stages, and this effect becomes still greater in the febrile stage. In numerous observations in dogs covering all stages of distemper and all degrees of diabetic tendency, precisely the opposite effect has been found. It is true that distemper is characterized by early failure of appetite and digestion. The resulting emaciation constitutes a very radical undernutrition treatment, and a similarity thus exists to Joslin's frequently quoted Case R (3), in which the emaciation of tuberculosis on a regulated diabetic diet evidently improved the assimilation. But the infection has been observed in dogs with definitely known tolerance, which continued for some time to take a diet close to the limits of tolerance. There have been other examples such as dog C3-77, 1 year old, weighing 10.5 kilos, and subjected on April 13, 1916, to the removal of all but $\frac{1}{2}\frac{1}{2}$ to $\frac{1}{3}\frac{1}{3}$ of the pancreas (estimated remnant 1 gram).[1] Glycosuria began immediately and by April 17 had reached 2.9 per cent. fasting. It then ceased as the first signs of distemper appeared in the form of conjunctivitis. The dog refused food and wasted away in the usual manner till killed on May 7, at a weight of 6.3 kilos. The pancreas remnant weighed 1.35 grams. The islands showed very slight vacuolation in a few cells, such as might persist from the initial glycosuria, but far less than would result from 3 weeks of active diabetes. The remnant was so small and the diabetic tendency so strong that sugar-freedom on fasting would have been impossible if the infection had introduced any aggravation, but the result seemed to be identical with that in a non-infected animal.

Pneumonia. Dog B2-18, after removal of $\frac{1}{1}\frac{3}{3}$ of the pancreas on December 2, developed moderate glycosuria on diets containing carbo-

[1] All operations were performed under ether anesthesia.

hydrate, in a cold environment. December 6 the dog was found to be unwell and febrile, but continued to eat the diet through the illness to December 9, and the glycosuria continued unchanged. Death occurred from double pneumonia on December 13. The autopsy urine contained 1.65 per cent sugar. The vacuolation in the pancreatic islands was similar to that of non-infected dogs at the same stage.

Dog C3-73 similarly underwent operation leaving $\frac{1}{10}$ to $\frac{1}{11}$ of the pancreas on April 3, and died of pneumonia on April 18. The final urine on April 17 contained heavy sugar, but death was apparently preceded by anuria.

Dog D4-72 was left with a remnant of $\frac{1}{9}$ to $\frac{1}{10}$ of the pancreas on January 12, and died of pneumonia on January 19. The appetite continued and the glycosuria was not appreciably changed with the onset of fever. Nothing was eaten after January 17. The autopsy urine still contained a trace of sugar. There was thus a diminution resembling the effect of ordinary fasting, not the great increase which usually accompanies infection in any severe human case.

Cat A1-93 was left with a remnant of $\frac{1}{5}$ to $\frac{1}{8}$ of the pancreas on January 21, and died of pneumonia on January 26. Because of refusal of food, there was only a transitory trace of glycosuria, as would be the case in a non-infected cat fasting after such an operation.

Other examples might be given in which infection failed to cause glycosuria when the removal of pancreatic tissue was not quite sufficient to produce it in a non-infected animal, and still others in which extreme prostration prevented glycosuria which must otherwise have occurred. In no instance was any evidence of aggravation of diabetes seen.

Pleurisy. Dog B2-22 had been used in another department for collection of leukocytes by intrapleural injections, and on December 8, $\frac{9}{10}$ of the pancreas was removed without knowledge of the existence of a large purulent pleurisy. Fever, malaise and other symptoms were found after the operation. The dog ate small quantities of bread and milk on December 9 and 10, and glycosuria of 2 to 3 per cent continued to December 12. Death occurred with sugar-free urine on December 13.

Subcutaneous abscesses. In connection with subcutaneous injections and other procedures a considerable number of abscesses have been observed in dogs with various degrees of diabetic tendency. The infections themselves have been of varying magnitude, from small collections, causing no systemic symptoms to large ones accompanied by depression, anorexia and fever above 105°F. The organisms present were sometimes identified as staphylococci, streptococci or mixed bacilli. The same rule held as above, namely, that glycosuria might cease with fasting and prostration, or in less extreme cases might continue unchanged, but a marked aggravation such as is familiar in human cases was never seen.

Infected glands. Dog D4-92 on February 5, 1917, was subjected to removal of all but $\frac{1}{8}$ to $\frac{1}{9}$ of the pancreas. Bread and soup were eaten on February 8, and 100 grams glucose added on February 9, still without glycosuria. Thereafter nothing was eaten and remarkable symptoms of confusion and ataxia appeared, increasing on February 12 to general convulsions and suggesting rabies. The dog was chloroformed on February 13, and the brain examination was negative for meningitis or Negri bodies. The autopsy otherwise was negative except for a little creamy pus found oozing from between the pectoral muscles, leading to caseous-appearing glands in and about both axillae. The type or origin of the infection was not determined. Glycosuria remained absent.

Rabies. Several partially depancreatized dogs died of rabies. One of these was dog B2-02, which, as previously mentioned (4), had been carefully studied and was known to have latent diabetes. No glycosuria resulted in any instance. The negative results were of interest in a condition attended with such pronounced nervous excitation, and in which convulsions may give rise to very marked hyperglycemia (5).

General peritonitis. This naturally involves cessation of glycosuria in most cases because of fasting and prostration. With sufficiently large experience, examples are encountered which indicate that the infection in itself does not alter the glycosuria. Some such were described previously (6), and the following have been observed since.

Dog B2-13. November 24, 1913, removal of $\frac{7}{8}$ of pancreas. There was glycosuria of 0.25 per cent in 60 cc. of urine following operation, and 0.2 per cent in 330 cc. after eating 150 grams meat on November 27. Otherwise there was fasting and freedom from glycosuria up to death from peritonitis on November 29.

Dog B2-21. December 4, 1913, partial pancreatectomy leaving a remnant of $\frac{1}{15}$ to $\frac{1}{14}$. After bread feeding on December 5, heavy glycosuria began, and continued to death from peritonitis on December 11. Bread was eaten daily to December 9. The autopsy urine was 100 cc., with 2.85 per cent glucose. The pancreatic islands showed the slight vacuolation proper to this early stage of diabetes.

Cat A1-82. December 19, 1913, removal of $\frac{7}{8}$ of pancreas. The cat refused food but acted well and cleaned her fur up to December 22, and died of peritonitis December 23. Glycosuria began with a faint reaction on December 21, rose to 2.5 per cent on December 22, and was 4 per cent in the last 55 cc. of urine on December 23. The pancreatic islands showed incipient vacuolation in a minority of cells.

Cat A1-87. January 8, 1914, removal of $\frac{3}{4}$ of pancreas. There was slight continuous glycosuria with very little eating from January 9 to death from peritonitis on January 12. The islands were free from visible vacuolation, as would be expected in a non-infected animal with such brief and mild diabetes.

Peritoneal and pancreatic abscesses. In addition to previous examples (6), the following may be mentioned.

Dog D4-65. December 21, 1916, an Eck fistula was unsuccessfully attempted, and some sutures were left on the veins. January 2, 1917, $\frac{7}{8}$ of the pancreas was removed. The dog was lively and immediately developed glycosuria on bread feeding. This ceased on January 8, and was restored by addition of 100 grams glucose daily. On January 10 emaciation, fever and weakness first became noticeable, but the diet was still eaten without change in the heavy glycosuria. With a change of diet to 1 kilo of beef lung on January 12 glycosuria immediately ceased. Beginning January 14 food was refused, and the dog was killed January 15, at a weight of 9.8 kilos as opposed to an original 13.5 kilos. A grape-sized abscess of creamy pus at the site of the Eck operation was the only discoverable cause of death. It had not altered the course of the diabetes from what is the rule with non-infected dogs under the same conditions.

Dog D4-57. December 7, 1916, removal of $\frac{3}{4}$ of the pancreas. The usual complete absence of diabetes was demonstrated thereafter. March 1, 1917, additional tissue was removed, possibly sufficient for mild diabetes. Malaise, fever and complete refusal of food followed. Glycosuria was absent on March 2, 3 and 4, but present just before death on March 5 to the extent of 0.8 per cent in 182 cc. of urine. The plasma sugar at this time was 0.625 per cent, CO_2 capacity 69.2 vol. per cent. Autopsy showed the pancreas remnant to be riddled with small abscesses, and though there was no necrosis the inflammatory injury had evidently brought on a severe degree of diabetes which would otherwise have been lacking. Infection has never been found to produce acetonuria or other evidences of acidosis in any animal.

Dog F6-14 was subjected to removal of about $\frac{3}{4}$ of the pancreas in three successive operations. January 31, 1918, an attempt was made to produce diabetes

by circulatory stasis of the remnant, as described in a later paper. Only slight and transitory glycosuria resulted on a diet of bread and soup with 100 grams glucose. February 8, operation showed an abscess containing about 5 cc. of creamy pus between the pancreas and the duodenum. The cavity was cleaned and stasis repeated. Glycosuria was still impossible to maintain, and on March 9 stasis was applied for a still longer time, no infection being found. Glycosuria was then continuous up to March 19 on bread diet with 100 grams of glucose, but ceased then on plain bread and soup feeding. March 20 the abdomen was again opened, and the pancreas was found buried in a large mass of adhesions, which when delivered outside and opened was found to contain a very large abscess. The pancreas remnant, which in its whole length formed one wall of the abcess, was much inflamed but not digested. Nothing was done except the cleaning up of the infection, and the tolerance continued exactly as before; i.e., glycosuria was absent on bread feeding and present with addition of 100 grams of glucose. On April 10 the abdomen was again opened, and a tiny abscess in the omentum appeared as the only remains of the previous infection. Stasis was again applied to the pancreas remnant, and the dog died within 24 hours, whether from infection or from pancreatic intoxication was undetermined.

The long history of this animal, with alternate presence and absence of a low grade infection, seems to prove that in this instance the infection had no important influence upon the tolerance.

Other examples of this sort might be given. There was particular interest in the cases in which diabetes was produced by inflammation instead of by simple resection, because of the supposed closer imitation of the clinical etiology. It was conceivable that inflammation might damage the islands in function as well as in structure, so as to render them more susceptible to toxic influences. The negative results raised a question concerning some fundamental difference between clinical and experimental diabetes, or a mere difference of constitutional reaction to infection on the part of man and animals. The above general observations sufficed positively to exclude any such marked aggravation of diabetes in animals as occurs regularly in human cases with the fever and intoxication accompanying infection. There remained the need of making a more exact test of the tolerance in experimental diabetes as influenced by infection, and this opportunity was afforded by the experiments with the gas bacillus reported in the next paper. These seemed to indicate that the difference between clinical and the experimental diabetes may be one of degree rather than of kind.

CONCLUSIONS.

1. The serious aggravation of diabetes, which occurs almost invariably in human cases in the form of a strongly increased tendency to glycosuria and acidosis, is never seen in dogs. Even when the infection is an abscess bordering or invading the pancreatic tissue, no influence is evident beyond that explainable by direct injury of parenchyma. This contrast between clinical and experimental diabetes is very marked, but according to the more exact tolerance tests in the succeeding paper it may represent a difference of degree rather than of kind.

2. Infection and fever have also no specific influence in diminishing the diabetic tendency of dogs. Care is necessary in interpreting such observations, in order not to confuse the direct influence of fever or infection with the consequences of fasting or prostration, which tend so strongly to suppress glycosuria in dogs. One suggestion of a constitutional difference between species may be found in the tendency of human patients to acidosis and of dogs to cachexia.

3. The aggravation of human diabetes is a reaction to intoxication rather than to fever, as shown by its occurrence in the afebrile stage of tuberculosis and by other evidence. The present observations concerning infectious fever, with the previous ones concerning the pyrexia of exercise in dogs, prove that no specific aggravation of diabetes or lowering of tolerance results from the metabolic alteration attendant upon elevation of body temperature in experimental animals.

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EXPERIMENTAL STUDIES IN DIABETES.

Series II. The Internal Pancreatic Function in Relation to Body Mass and Metabolism.

6. Gas Bacillus Infections in Diabetic Dogs.

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In the course of this diabetic research four dogs died of gas bacillus infection. Two of these instances were merely post-operative peritonitis, with the gas bacillus predominating.

Another was dog E5-74, a bulldog mongrel, aged 5 years, in splendid condition, weighing 20.7 kilos. July 3, 1917, two-thirds of the pancreas were removed, and most of the remnant was cut off from duct communication.[1] On July 20, the remnant was subjected to circulatory stasis for $1\frac{1}{2}$ hours. Glycosuria was present on bread diet with addition of 150 grams of glucose daily up to July 30, when it ceased, and the dog was turned loose in the yard with other dogs on bread diet. The behavior meantime was normal. About August 5, swelling in the neck became noticeable and the dog was slightly depressed. August 7, the swelling was much larger, and a deep abscess was opened surgically, releasing a considerable quantity of thick bloody pus containing gas bubbles. Cultures from some of the necrotic debris gave a pure growth of B. aerogenes capsulatus. Death occurred August 9, after still greater invasion of the neck. There was no glycosuria or vacuolation of pancreatic islands, though both these conditions might have been prevented by the terminal emaciation and cachexia.

Dog B2-49, a female mongrel aged 3 years, in medium nutrition at a weight of 25.4 kilos, underwent partial pancreatectomy on March

[1] All operations were performed under ether anesthesia.

27, 1914, leaving a remnant of $\frac{1}{4}$ to $\frac{1}{5}$ about the main duct. As previously mentioned (1), prolonged carbohydrate over-feeding was used in the attempt to break down tolerance, 300 or 400 grams glucose being added to the diet of bread and soup daily. There was neither glycosuria, diarrhea nor any evident ill-health, until the animal was unexpectedly found dead on May 9. There were adhesions in the right pleura, from supposedly sterile intrapleural injections in another department long before the animal was taken for diabetic work, and these probably furnished the start of the infection. Gas bacilli were found abundantly in smears and cultures from the principal viscera, seemingly alone. The greatest change was in the spleen, which was blown up to resemble a lung. The pancreas remnant was normal and free from vacuolation. In other words, neither the prolonged sugar feeding nor the infection produced any change in either islands or acini in this non-diabetic animal.

A study of gas bacillus infections was in progress at this time under the direction of Dr. Carroll G. Bull. As gas bacillus infections are rare in dogs, it was decided to follow up the above accidental observations by experiments upon diabetic dogs with a view to two questions; first, whether such animals are abnormally susceptible to such infections by reason either of the excess of circulating sugar or a specific diabetic lowering of resistance; second, whether an aggravation of the diabetes is demonstrable by such infections. The conditions were favorable for both problems; for the first problem because the growth of the gas bacillus is notably favored by the presence of sugar, and some test was thus afforded of the theory of excess of sugar as the cause of diabetic susceptibility to infection; for the second problem because of the proof (2) of the production of a soluble toxin by the gas bacillus, so that a systemic effect capable of influencing the diabetes might be expected from a local infection. Accordingly experiments with intramuscular injections of pure cultures of the Welch bacillus were performed upon three diabetic dogs. The dosage used was intended to produce the maximum possible local effects and general intoxication without excessive prostration. Still larger doses might have overwhelmed the animals suddenly and completely, but would thus have demonstrated nothing of value for either bacteriology or diabetes.

TABLE 1.

Dog E5-88. Male; Welsh terrier mongrel; old but strong, in excellent nutrition; weight 11.25 kilos. August 24, 1917, removal of pancreatic tissue weighing 18 grams. Remnant about main duct estimated at 1.6 grams ($\frac{1}{1}^{1}_{2}$ to $\frac{1}{1}^{1}_{3}$). The diabetes was checked by undernutrition and fasting, so that at the time of the experiments the dog weighed 9.5 kilos and took a bread and soup diet with very slight glycosuria.

DATE	RECTAL TEMPERATURE	URINE		REMARKS
		Volume	Glucose	
1917	°C.	*cc.*	*per cent*	
September 10.....		660	0.3	Bread and soup diet
September 11.....	38.7	450	0.4	
September 12.....		510	Trace	At 10:45 a.m. injected 0.1 cc. per
10:00 a.m.....	38.8			kilo of broth culture of Welch ba-
1:15 p.m.....	39.7			cillus intramuscularly right thigh.
3:30 p.m.....	40.7			Marked local edema and swelling.
5:30 p.m.....	40.4			Dog depressed; ate nothing
September 13.....				Thigh still swollen. Dog unwell but
9:00 a.m.....	39.7	880	2.6	took entire diet, part forcibly
September 14.....				9:30 a.m., injected 0.3 cc. per kilo of
9:00 a.m.....	38.3	670	2.8	broth culture of Welch bacillus in-
				tramuscularly left thigh. Much
				local swelling. Dog ill and fever-
				ish; refused diet but ate a little meat
September 15.....	39.8	320	1.4	Refused all food
September 16.....		425	0.3	Refused all food. Great edema and
				crepitation, extending into scrotum
September 17.....		510	Trace	Refused food
September 18.....		650	0	Refused food
September 19.....		420	0	Refused food
September 20.....		400	Trace	Ate a trifle of meat and bread
September 21.....	39.5	450	0	Ate very little of meat
September 22.....		480	Trace	Ate more meat. Much swelling and
				gas in leg
September 23.....		460	0.6	Acting better. Ate more meat
September 24.....		400	1.2	Ate some bread and meat
September 25.....		500	2.1	Ate full diet
September 26.....		700	2.9	Ate full diet
September 27.....		630	3.4	Ate full diet
September 28.....		610	3.1	Ate full diet
September 29.....	38.8	1200	2.2	Both thighs have discharged necrotic
				material, leaving granulating ulcers.
				Dog lively and vigorous

In the first experiment (table 1) glycosuria practically ceased with the anorexia accompanying infection on September 12, as usual with dogs and in contrast to the usual aggravation of symptoms in human patients with infection. Nevertheless a lowering of tolerance was shown by the heavier glycosuria when the diet was taken on September 13. Illness and fasting again resulted in sugar freedom after the injection of September 14, but a more marked lowering of tolerance was evident in the glycosuria from meat alone on September 22 and 23, and the heavier glycosuria thereafter on the regular bread diet.

Dog E5-89. Male; mongrel; age 3 or 4 years; good condition; weight 14 kilos. August 24, 1917, removal of pancreatic tissue weighing 25 grams. Remnant about main duct estimated at 1.6 gram ($\frac{1}{16}$ to $\frac{1}{12}$). Severe diabetes being thus produced, the glycosuria was raised to a maximum by a diet of bread and soup with 100 grams of glucose daily.

September 7, at a weight of 12.6 kilos, 0.25 gram additional pancreatic tissue was removed for microscopic examination.

September 14, at the same weight, 0.1 cc. broth culture of Welch bacillus per kilo was injected intraperitoneally, in order to test whether under these conditions of maximum glycosuria and hyperglycemia infection would be possible. The rectal temperature rose within an hour to 39.4°C. After 6 hours it was 39.5°, and the next morning 39.6°. It then subsided, and after one day of slight malaise the dog continued to eat his diet. The glycosuria continued unchanged except for a diminution on the one day of anorexia.

September 24, an injection of 0.3 cc. of broth culture per kilo was given intramuscularly in one thigh. The usual local and general symptoms occurred in intense form. September 29, with very large swelling and gas formation present in the leg, a blood culture was taken and proved negative. The dog regained a little appetite, taking small amounts of meat and bread daily, but great anemia was shown by blood examinations, the corpuscle volume being only 10 to 12 per cent. Death occurred October 5. Glycosuria remained heavy throughout, including the autopsy urine. The gross autopsy showed no visceral changes suggestive of gas bacillus invasion. Cultures of blood and tissues were also negative for this organism.

The pancreas remnant, normal in appearance and consistency, weighed 1.7 grams. Microscopically, the tissue removed August 7 showed a very early stage of vacuolation of islands. The remnant at autopsy showed a late stage of the process; islands were scarce and small, and the great majority of the cells (probably all of the beta cells) were maximally vacuolated.

In this experiment the production of a general infection with the gas bacillus proved impossible notwithstanding the severe diabetes

and intense glycosuria. The intraperitoneal injection failed entirely. The intramuscular injection caused extensive sloughing which destroyed most of the musculature of the limb, but death resulted only from the immediate and subsequent toxic effects and not from systemic invasion.

Dog E5-90. Male; mongrel, age 3 or 4 years; medium nutrition; weight 10.25 kilos. August 24, 1917, removal of pancreatic tissue weighing 20.3 grams. Remnant about main duct estimated at 1.6 gram ($\frac{1}{1}$ to $\frac{1}{4}$). On bread diet there was a diminishing glycosuria which ceased August 31, probably because of hypertrophy of the pancreas remnant, which subsequently at autopsy was found to weigh 5.4 grams. Beginning September 8 the addition of 100 grams glucose restored a heavy glycosuria, and the tolerance was brought down so as to produce a permanent mild diabetes. During October sugar freedom was maintained on a diet of 500 grams lung and 100 grams suet, except for occasional days on which it was proved that bread and soup diet would promptly bring back a mild glycosuria.

TABLE 2.

BLOOD			URINE						
Plasma sugar			Volume			Glucose			REMARKS
October 25	November 1	December 18	October 25	November 1	December 18	October 25	November 1	December 18	
per cent	*per cent*	*per cent*	*cc.*	*cc.*	*cc.*	*grams*	*grams*	*grams*	
0.164	0.133	0.122				0	0	0	Before injection
0.333	0.400	0.384	5	41	14	0.034	0.450	0.320	End of 1st hour
0.323	0.400	0.500	14	42	50	0.080	1.150	2.000	End of 2nd hour
0.213	0.216	0.455	95	14	26	Faint	0.360	0.680	End of 3rd hour
0.081			400	150	30	Slight	0.690	0	Next morning

October 25, an intravenous glucose tolerance test was performed, by injection of 25 cc. of 10 per cent solution of Merck anhydrous glucose every 15 minutes (1 gram per kilo per hour, on 10 kilos weight) for 3 hours, according to the method described elsewhere (3). Catheterization was performed and blood samples taken before the first injection and at hourly intervals thereafter as shown in table 2.

October 26, 4 cc. of a heavy broth culture of the Welch bacillus were injected intramuscularly in the right thigh. The rectal temperature rose to 41.1°C. that evening and was 39.6° the next morning. The dog refused food and there was no glycosuria. By October 31 there was partial recovery and part of the diet was eaten. The weight had fallen from 10 kilos to 9.75.

November 1, an intravenous glucose test was performed, identical with the dosage on October 25. A lowering of tolerance was indicated by both the blood and urine analyses.

November 14, 4 cc. of the gas bacillus culture were injected in the other thigh. Local edema, gas formation and necrosis occurred as before, but the general symptoms were less. The temperature on the morning of November 15 was 38.8. The dog ate well and showed a spontaneous glycosuria of 2.15 per cent in 340 cc. urine. The following day it was 0.48 per cent in 530 cc. urine, and then disappeared.

Later the dog was unwell and ate poorly, probably on account of secondary infection of the sloughing area in the leg. No further glycosuria developed, and by December 18 the animal was again in good general health at a weight of 10 kilos, though a large open ulcer was still present.

December 18, the animal was given the same intravenous glucose injections as before. A reduced tolerance was still indicated, either because of the ulcer or because the lowering due to infection was permanent, as it is in many human cases.

An accidental or spontaneous fall of tolerance is probably excluded by the fact that the dog was kept on the lung and suet diet till March 27 without glycosuria. He was then used for other experiments, and no further tolerance test was made.

CONCLUSIONS.

1. Intramuscular injections of pure cultures of *B. aerogenes capsulatus* produced local necrosis and gas formation in partially depancreatized diabetic dogs. Systemic or peritoneal infection was not obtained. The observations failed to indicate any lowering of resistance in these animals due either to the diabetes itself or to the excess of sugar in the body fluids. The latter point is further emphasized by the fact that the reactions were essentially similar in the first dog with mild glycosuria, in the second dog with heavy glycosuria, and in the third dog free from glycosuria. These results agree with the general experience that such animals ordinarily bear operations well and their wounds heal normally.

2. A lowering of tolerance by infection was demonstrable both by feeding and by intravenous glucose tests. Though this influence is less in animals than in human patients, the difference seems to be one of degree rather than of kind.

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THE EARLY STAGES OF TABANIDÆ (HORSE-FLIES).

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PLATES 1 to 15.

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INTRODUCTION.

The Tabanidæ are a group of blood-sucking insects which, though of less importance to medicine than mosquitoes and certain groups of flies, are nevertheless of sufficient interest to the student of animal pathology to warrant a closer study and a completion of our meager knowledge of the life history of these insects. As the group consists of many species spread over all parts of the world, it is natural that the work done hitherto has scarcely advanced beyond the systematic and descriptive stage; and the lack of information on development and early stages is especially felt by experimental science. In fact, although Tabanidæ play a considerable part in transmitting diseases of domesticated animals, and in spite of the trouble which their presence in large numbers causes to horses and cattle even under normal conditions, our knowledge of their life history is very incomplete compared with that which we possess of other groups of blood-sucking insects; for instance, Culicidæ. Only recently the study of tabanid life histories has been undertaken from the point of view of their economic importance, by King, British Government Entomologist in the Anglo-Egyptian Sudan, Mitzmain in the Philippine Islands, and others. However, these authors practically ignore previous or contemporary literature on the subject of their investigation. Thus Mitzmain states that the literature on the subject is meager and quotes only three publications. When rediscovering Graber's organ in the larvæ studied by him, he assumes that the organ is peculiar to this species or has been overlooked by the previous authors. The Zürich Index (Concilium Bibliographicum), however, contains reference to several publications of European authors on this organ.

I have undertaken a review of the literature on the subject because the entomological literature is especially difficult of access, being scattered over a number of small periodicals appearing in different languages. Field and experimental work on entomological subjects is often done at a great distance from large libraries, and it would be an advantage to the individual worker, especially in the tropics, to

have all the facts on a definite subject concentrated in one publication. I have therefore decided to give not only a summary of the literature with the bibliography, as is usually done in text-books, but also a complete report of all the facts found in these publications, with very little change in the wording, literal quotation of all authentic descriptions, and with the reproduction of practically all the existing illustrations on the subject, sufficiently accurate to make the consultation of the originals unnecessary. By this means I hope to relieve the worker on the subject in question of the necessity of consulting libraries, except the future monographer of the whole group, who will prefer to go back to the original publications. On the other hand, as a full bibliography is given, in case of uncertainty about any detail, the originals can always be consulted and compared with this report.

There is, however, another consideration of some importance which has guided me in preparing this review; that is, the desirability of having, generally, all the facts on one subject of research concentrated in one language, preferably English. The diversity of languages is a great hindrance for the progress of science, and from the point of view of methodical unification, specialists in any branch of science should be encouraged to concentrate all the literature on their own subject in the language which they prefer to have used. Having given considerable time to the study of this question, I cannot see any serious objection to the more general adoption of English in science. It is to be hoped that the temporary disadvantages to the non-English-speaking scientists, resulting from its adoption would be made up by the advantages of a great progress in science which would undoubtedly follow. It is for the scientists of English-speaking countries to pave the way, by conquering the field systematically, subject after subject, and to facilitate the production of new work by a complete assimilation of work previously done in foreign languages.

I wish to express my indebtedness to the authorities of the American Museum of Natural History and of the Philadelphia Academy of Natural Science for giving me liberal access to their libraries and collections, and to Dr. Theobald Smith, Director of the Department of Animal Pathology under whose guidance this work was rendered possible.

In grouping the material I have as far as possible followed a systematic arrangement, by bringing all the facts which could be referred with certainty to a definite species under the heading of that species, even where the facts appear to apply to a whole genus or to tabanids in general. In each species the egg stage is first treated, followed by the larval and pupal stages. Results obtained by one author in one paper have, consequently, often been separated and rearranged. On the other hand, no attempt has been made towards extensive generalization; the general statements on the family Tabanidæ and the more important genera are only such as are found already in the literature, and are not to be considered as final or as expressions of the author's opinion.

In the arrangement of the plates a slightly different plan has been followed, as it seemed desirable to facilitate the comparison of corresponding stages. I have therefore figured on Plates 1 and 2 all the existing illustrations of the egg stage of different species; on the following plates the larvæ and larval structures; following these the pupæ; and finally pupal structures, these being of especial systematic value. With this arrangement the different stages of the same species are necessarily separated and figured on different plates. In the details, the arrangement has been such as seemed most convenient. Occasionally an exception to the general order has been made and a group of figures illustrating one species or genus appears on the same plate.

HISTORY OF INVESTIGATIONS.

In 1760 the Swedish naturalist Degeer published a paper, in the transactions of the Swedish Academy of Science, on the larval and pupal stages of the European horse-fly, *Tabanus bovinus*, which he found to be terrestrial in habit, and which he described in a paper bearing the title *Bromsarnas ursprung* (The origin of horse-flies). Degeer was a contemporary and compatriot of Fabricius, the first great entomologist, and both continued the work of Linné. It is stated by various authors that Fabricius' works also contain data on the early stages of Tabanidæ, though very few, but I have not been able to find such data in the *Systema Entomologiæ* (1775) or in the *Entomologia Systematica*. The statement that the larvæ of *Chrysops* are found in the ground cannot have been made by Fabricius, in this form, as the genus *Chrysops* was first established by Meigen (1803). If Zetterstedt makes this statement in *Diptera Scandinaviæ*, it is possible that it is from verbal information or from some later writings of Fabricius. Zetterstedt seems to doubt it, stating that he has seen large numbers of newly emerged *Chrysops* near the margin of a lake, which would indicate an aquatic habitat. The Swedish school for a considerable time dominated entomological work in all countries. Degeer's observations are reprinted in his *Mémoires pour servir à l'histoire des insectes*, which appeared in French in 1775 and also in German translation (Goeze, 1782). These observations are given without change by Macquart in *Histoire naturelle des insectes. Diptères* (1834), and by Westwood in England, the illustrator of the *Arcana Entomologica*, in his paper Introduction to the modern classification of insects (1840). To the same school belongs Wahlberg, who in 1838 published, in Swedish, accounts of the larval stages and mode of life of many Diptera, which also contain, according to Brauer, notes on tabanid larvæ living inside lepidopterous larvæ, probably in a pseudoparasitic way. In one of his papers on this subject I could not find any reference to Tabanidæ.

The progress of entomology consisted chiefly in a greater specialization as to definite orders which were systematically studied and

described, and in the greater attention which was given to the larval stages. A new school of investigation on this subject arose in the German-speaking countries with Vienna as its center, where Schiner had become an authority on the Diptera of Austria-Hungary, and where Brauer devoted himself to a comparative study of insect metamorphosis, particularly of dipterous larvæ. It is to be noted that Scholtz, in 1848, in Breslau, on an excursion in which von Siebold also took part, discovered some tabanid larvæ, indicating an influence of the growing science of general zoology on this subject. In 1854 Mann discovered oviposition in *Tabanus autumnalis*, and Kollar, at the Vienna Museum, observed the eggs of various other species of *Tabanus*, stating that since Degeer nothing had been known about the development of these insects. In the following decades great progress was made in the knowledge of dipterous and other insect larvæ, chiefly through the Vienna school. In 1868 one of Brauer's students, Marno, found the larvæ of *Hexatoma pellucens*, a tabanid not rare in Austria, this being one of the first instances known of an aquatic larval stage in this family. This larva was described more fully in 1883 by Brauer himself. In 1869 Brauer was able to describe the larva and pupa of *Hæmatopota pluvialis* (*Regenbremse*), which is terrestrial, giving at the same time a careful drawing of the mouth-parts with correct interpretation of all the details. At about the same time Brauer was working on the classification of dipterous larvæ. The larva of *Hæmatopota pluvialis* was, however, described also by Perris in France in 1870, who found it in rotten pine wood, and gives a figure showing the fine striations which are not shown in Brauer's figures; and the same larva was described in 1875 by Beling, inspector of forests in the Harz mountains, both apparently independent of Brauer's studies. Beling was also the first to describe the pupa of *Chrysops*, in 1882. On the other hand, von Friedenfels, who found the larva of *Tabanus autumnalis* in the salt lakes of Siebenbürgen, belonged to the Vienna naturalist group, and the larva which he had supposed to be an annelid was identified by Brauer. The results of all these investigations are briefly summarized by Brauer in 1883 in the third volume of *Die Zweiflügler des Kaiserlichen Museums zu Wien*, in which the larvæ of *Tabanus solstitialis*, *spodopterus*, and *bromius*, and the larva of *Hexatoma pellucens* are described.

During the period just mentioned the microscopic study of lower organisms had made continuous progress and the anatomical investigators soon began to discover favorable objects of research in the histology of insects. The study of sense organs of lower animals had since Johannes Müller become a favorable subject of research. On the other hand, the native fresh water fauna furnished the most varied and suitable objects for microscopic study, and this fact led to repeated observations on tabanid larvæ from the point of view of general zoology, the determination of the species at hand being omitted with an almost disconcerting regularity. We have, however, noted that tabanid early stages were observed by Scholtz in Breslau in 1848, and even the species was identified, as adults had hatched from the pupæ collected. In 1878 Graber, in Czernowitz, Austria, in his studies on the sense organs of insects, discovered a peculiar otocyst-like organ in a *Tabanus* larva, called Graber's organ by later authors, a discovery which he connected with that of similar organs in the larva of *Ptychoptera* by the zoologist Grobben. His paper called forth an article by Krauss in 1879, a pupil of Brauer in Vienna, according to whom this organ had already been demonstrated by Brauer in the tabanid larva in his zoological course. Krauss also declared the larva defined by Graber as a fly-maggot to be a *Tabanus* larva, and to belong to *Tabanus autumnalis*. Whether the species was really this or a similar one, no one can say at present. However, to Graber belongs the credit of the independent discovery of this organ and its minute description. In a later work (1882) on the chordotonal organs of insects, Graber figured another tabanid larva with many anatomical details, demonstrating in it the chordotonal organs which had been discovered by Leydig in *Corethra plumicornis*. These discoveries, especially the otocyst-like organ, gave rise to scientific controversy, Lécaillon in France (1905 and 1906) assuming that the organ must in reality be a gland, while Paoli (1907) in an extensive work undertakes to prove that it is not an auditory, but a sound-producing organ. At the same time Paoli advanced an interesting theory about the manner in which the organ is developed, though without reference to phylogeny.

With Lécaillon and Paoli an influence of economic entomology, especially of agriculture, is notable in stimulating research, unques-

tionably a reaction on Europe from America. In America research on the early stages of Tabanidæ begins with Walsh (1863), who belongs entirely to the old type of naturalist like Westwood and others, describing facts of nature half as pioneers of discovery in strange countries, half as pioneers of religion, and filled with a deep admiration of the wisdom manifesting itself in creation. He is contemporary with Osten Sacken, the first systematic specialist of American dipterology, who published his Prodromus to a monograph of North American Tabanidæ. In Walsh's publication of previous work only Degeer is referred to, and Walsh believes that his larva, which was proved later to be that of *Tabanus atratus*, was the first instance of an aquatic tabanid larva. In fact, though the observations of Wahlberg, Zetterstedt, Scholtz, and Kollar were not known to Walsh, these authors give only indications but no full evidence of aquatic habits of life of the larvæ observed by them. While a member of the Boston Natural History Society, Walsh lived for some time in Illinois, where tabanids were numerous and annoying, so that the economic side of the subject inevitably received attention. We may say that with him begins the American or economic school of investigations on this subject. How curiously the economic considerations were mixed with a profound confidence in the wisdom of nature may be seen from the following quotation from his paper, with which few modern investigators will be found to agree, except perhaps the last sentence:

"The scheme of the creation is perfect, and nature is never at fault. It is only when nature's system is but half understood that we heedlessly complain of its imperfections. We blame the house-flies for annoying us, and fail to see that in the larva state they have cleared away impurities around our dwellings, which might otherwise have bred cholera or typhus fever. We execrate the blood-thirsty mosquito, and forget that in the larva state she has purified the water, which would otherwise, by its malarial effluvia, have generated agues and fevers. In all probability, when we rail at the *Tabani*, which torment our horses in the summer, we are railing at insects which, in the larva state, have added millions of dollars to the national wealth, by preying upon those most insidious and unmanageable of all the insect foes of the farmer—subterraneous root-feeding larvæ."

The larva of *Tabanus atratus* is redescribed by C. V. Riley in 1870 in the second of his Missouri Reports, this being the first publication

on a tabanid larva issued from an agricultural institution. Riley apparently was interested in all insects and their development, and in fact included in his Reports also some insects of no economic importance.

A great step in advance was made when in 1895, Hart, in Illinois, probably interested by Walsh, who had lived there, but probably also not without some knowledge of the studies of the Vienna school, undertook to investigate the entomology of the Illinois River on a large scale. He described large numbers of new dipterous larvæ, and incidentally a number of tabanid larvæ, notably those of *Chrysops vittatus, Tabanus stygius, nigrescens, lineola*, and *costalis*, which were not known before, and giving a preliminary classification by which these larvæ could be separated. Hymenopterous egg parasites of *Tabanus*, previously seen by Kollar, were then for the first time exactly described by Ashmead, who cooperated with Hart. I do not think that Hart could have achieved his results without a knowledge of Brauer's monograph, which had, in fact, appeared ten years before, and which, as stated, contains references to most of the previous work. But as he, apparently following Agassiz's principle, "Study nature, not books," makes no mention of the previous literature on this subject, I assume this to be the reason why later authors, Hine, and even Lécaillon, have lost the thread of tradition pertainng to their subject, and why important results such as the descriiptions of numerous larvæ and the discovery of Graber's organ have been overlooked by American and European authors. Shortly afterwards, in 1899, the insect volume of the Cambridge Natural History, by Sharp, appeared in England, which also does not mention the literature, but contains the description of an unknown tabanid larva, referred to by Sharp as *Tabanus* (*?Atylotus fulvus*).

In America new discoveries of tabanid early stages were made by Hine, who, continuing Osten Sacken's work in this field, has become the leading authority on the Tabanidæ of North America, especially from the systematic point of view. In 1903 he described the life history of *Tabanus vivax;* this paper was followed by a publication on the Tabanidæ of Ohio, which also contains notes on the early stages. It is worthy of note that Hart's as well as Hine's first publications were not issued by economic but by scientific institutions (Illinois Biolog-

ical Survey, Ohio Naturalist, Ohio Academy of Science); later the connection with agriculture was established, first in Hine's Report on the Tabanidæ of the Gulf Coast (1903), then in his Habits and life histories of some flies of the family Tabanidæ (1906), in which the early stages of *Tabanus lasiophthalmus*, and some others, are given for the first time. The Tabanidæ, long known as a stock pest, were at about that time suspected of carrying infectious cattle diseases (anthrax and surra), as was evident from Salmon and Stiles' Emergency report on surra published in 1902 in connection with a small outbreak of surra in the United States following the importation of Indian zebu cattle. The interest taken by entomologists in Hine's work called forth some smaller publications in America, as in 1908 that of Walton, containing descriptions of the early stages of *Goniops chrysocoma*, a peculiar species, and in 1909 that of Brimley, with notes on the early stages of several other Tabanidæ, one of them *Tabanus fronto*, being terrestrial in habit. On *Goniops chrysocoma*, under the auspices of the United States Department of Agriculture, a more detailed study was made by McAtee in 1911.[1] On the other hand, in methods of control of Tabanidæ, some progress was made by the work of Portschinsky in Russia, in 1908; Lécaillon in 1905 in France; and Paoli in 1907 in Florence, also undertook studies on Tabanidæ, which apparently were stimulated by work already done on this subject in America, Lécaillon referring to Hart, and Paoli, while resuming the problem of Graber's organ, working at an agricultural school where he undoubtedly received Hine's publications. Lécaillon's work (1905), which acquaints us with the egg-laying habits of *Tabanus quatuornotatus*, cites the observations on this species made by Kollar in 1854. The progress made in the meantime by the Vienna school is, however, ignored by him, which we understand if we realize that the wars of 1866 and 1870 had destroyed cooperation to some extent. The only new author cited by Lécaillon is Hart, who, as we have stated, did not quote the literature. At the same time, Lécaillon (1905) and Hine (1906) are perhaps the first writers in whom the influence of medicine on entomology is perceptible. The part that insects play in the transmission of disease, the theory of which is intimately con-

[1] Specimens of the larva of this species had been found before by Pergande.

nected with the discoveries of Theobald Smith (1893), Bruce (1895), Ross (1897), Grassi (1900), and Reed (1901), had attracted attention to all blood-sucking insects as possible carriers, and the influence on the study of Tabanidæ showed itself in the progress of knowledge of their early stages. In the following period extending up to the present, studies on these have been made largely under the auspices of medical entomology. The progress resulting is considerable, the number of publications on the subject within the last fifteen years being about equal to that of papers published previous to 1900.

To the interest aroused in the study of Tabanidæ from the medical point of view we owe in the first place a knowledge of life histories of tabanids of tropical countries, chiefly Africa and India, where the importance of these flies as carriers of disease is paramount. In 1908 King was sent by the British Government to the Sudan in charge of economic entomology in the Wellcome Research Laboratory in Khartoum. He goes into the subject more extensively than any of the previous authors, working out the life history of the black African horse-fly, *Tabanus biguttatus* (1908) and of *Tabanus ditæniatus*, *tæniola*, *kingi*, and *par* (1910), succeeding for the first time in causing tabanids to oviposit in captivity, and giving minute descriptions of larval structures, which appear cumbersome but may well serve to separate the species described from others not yet known. At about the same time we learn of tabanid larvæ in India from Maxwell-Leffroy and Howlett, Indian insect life (1909), a work, however, written more from the point of view of agricultural interests, while Baldrey and Mitzmain studied tabanids in captivity with the immediate object of determining their part as carriers of disease. Baldrey (1911–12) had *Tabanus orientis* oviposit in captivity, but made no observations on the larvæ. Mitzmain has made a very complete investigation on the life history of a single tabanid species, *Tabanus striatus* (1913). Mitzmain worked in the Philippine Islands and demonstrated experimentally the transmission of surra (1913) and anthrax (1914) by this species. His omission of reference to previous literature has been commented upon, but as his work was done far away from large libraries he was naturally handicapped in this respect. While Mitzmain raised his flies entirely in captivity, Bainbridge and Fletcher (1914) reported the oviposition of the same species in the

free-living state. The text-book of medical entomology by Patton and Cragg (1913), who worked in Madras, again gives descriptions of egg and larval stages of various Indian tabanids, with information on habits, methods of rearing, etc., giving also for the first time a more complete idea of work previously done. The organ discovered by Graber (1878) is illustrated and discussed by these authors; Paoli's work on the same subject, however, seems to have remained unknown to them. At about the same time (1914) Lutz in Rio de Janeiro published the first meager observations on early stages of tabanids in Brazil, having found larvæ of *Tabanus* (*Neotabanus*) *ochrophilus* and *triangulum*, and of one undetermined species. The studies of Neave in the following year, 1915, furnish abundant information on early stages of African tabanids, and new studies on South American tabanid larvæ were published by Bodkin and Cleare in 1916. Some new species of egg parasites have also been described in recent years by Crawford from King's material, and by Girault (1916) from *Tabanus* eggs from Dallas, Texas.

The various text-books on medical entomology and related subjects which have appeared recently, besides those quoted by Bainbridge and Fletcher and by Patton and Cragg, those published by Göldi (1913), Herms (1915), W. A. Riley and Johannsen (1915), and Grünberg (1907), also Brumpt's Précis de parasitologie (1910), contain a good deal of information on tabanid early stages, including also quotations from previous literature.

While most of the recent work has been done by British and American authors, new and accurate studies on the oviposition of *Tabanus quatuornotatus* have been made by Lécaillon (1911), and another paper by Picard and le Blanc (1913) reports the early stages of another species. In the latter year there also appeared a paper by del Guercio on the tabanids of the rice fields of Bologna, which, however, contains little of scientific value.

The complaint made by some writers of the meagerness of our knowledge of the life history of this group, notably by Göldi, will probably soon be unfounded, if our knowledge progresses as rapidly as it has in the last few years.

South America, which is extremely rich in interesting tabanids, and Australia, where until now apparently no work has been done,

should yield many new discoveries. On the other hand, European investigators have neglected the Tabanidæ somewhat, and it would seem desirable to reinvestigate the larvæ of the common *Tabanus bovinus*, which since their discovery by Degeer in 1760 apparently have not been found again.

CHRONOLOGY.

1760. Degeer bred *Tabanus bovinus* from terrestrial larva.
1775. Fabricius' Systema entomologiæ, said to contain notes on *Chrysops* larva.
1776. French translation of Degeer's Mémoires.
1798. Fabricius' Entomologia systematica.
1834. Macquart cited the observations of Degeer.
1838. Wahlberg, in Sweden, published notes on the larvæ as semiparasites.
1840. Westwood cited the observations of Degeer.
1842. Zetterstedt's observations on *Chrysops*.
1848. Scholtz observed pupæ of three tabanid species near Breslau.
1854. Kollar published Mann's observations on oviposition of *Tabanus*.
1863. Walsh, in Boston, described the larva of *Tabanus atratus* (aquatic).
1868. Marno, in Vienna, found the aquatic larva of *Hexatoma pellucens*.
1869. Brauer described the terrestrial larva of *Hæmatopota pluvialis*.
1870. Perris, in France, independently described the same larva.
1870. Riley, in America, redescribed the larva of *Tabanus atratus*.
1875. Beling, in Germany, described (third) the larva of *Hæmatopota*.
1878. Graber discovered an otocyst-like organ in the tabanid larva.
1879. Krauss claimed the discovery for Brauer.
1880. von Friedenfels found aquatic larva of *Tabanus autumnalis* in salt lakes in Siebenbürgen.
1882. Beling described the pupa of *Chrysops relictus*.
1882. German translation of Degeer's Mémoires.
1882. Graber described the chordotonal organs of *Tabanus* (*Chrysops?*) larva.
1883. Brauer gave notes and illustrations on various tabanid larvæ and briefly summarized the literature.
1895. Hart, in Illinois, described several American species of tabanid larvæ, notably *Tabanus stygius*, *lineola*, *costalis*, and *Chrysops vittatus*.
1899. Sharp, in The Cambridge Natural History, illustrated larvæ of *Tabanus* (*?Atylotus fulvus*).
1903. Hine, in Ohio, described the larva and pupa of *Tabanus vivax*.
1903. Hine's Monograph on the Tabanidæ of Ohio, with notes on life histories, containing also the first notes, and on oviposition in *Chrysops*.
1904. Hine's studies at the Gulf Biological Station.

1905. Lécaillon, in France, gave detailed studies on the oviposition of *Tabanus quatuornotatus*.

1903–06. Hine's Gulf Biological Station Reports, containing new data on oviposition.

1906. Hine's Report to the United States Department of Agriculture, describing early stages of *Tabanus lasiophthalmus*, *sulcifrons* (in part), etc.

1907. Paoli, in Florence, continued the work of others on Graber's organ.

1908. Portschinsky, in Russia, summarized the previous knowledge of tabanid life histories, as an aid in the study and control of these insects.

1908. King, in Khartoum, described the early stages of *Tabanus biguttatus*.

1908. Walton, in Pennsylvania, described the egg and larva of *Goniops chrysocoma*.

1909. Brimley, in North Carolina, published notes on the larvæ of *Tabanus fronto* and other species.

1909. Maxwell-Leffroy and Howlett, in Indian insect life, gave notes on the larval habits of Indian tabanids.

1910. McAtee, in Washington, described further stages of *Goniops*.

1910. King, in Khartoum, published the life histories of *Tabanus par*, *tæniola*, *ditæniatus*, and *kingi*, securing oviposition in captured specimens.

1911. Lécaillon gave additional studies on *Tabanus quatuornotatus*.

1911–12. Baldrey observed oviposition of *Tabanus orientis*.

1913. Mitzmain published the life history of *Tabanus striatus*, the carrier of surra in the Philippines.

1913. del Guercio, in Italy, reported on the larva of *Tabanus ignotus*.

1913. Picard and le Blanc, in France, observed the larva of *Tabanus cordiger*.

1913. Patton and Cragg published a text-book of medical entomology, containing information on the larvæ of several Indian tabanids.

1914. Bainbridge and Fletcher, in India, gave additional notes on *Tabanus striatus*.

1914. Lutz, in Rio de Janeiro, described the larvæ of three Brazilian tabanids.

1915. Neave presented abundant data on the larval and pupal stages of African tabanids.

1915. Riley and Johannsen, published a text-book, containing a few new illustrations.

1916. Girault described a new egg parasite (*Phanurus emersoni*).

1916. Bodkin and Cleare published notes on the early stages of tabanids in British Guiana.

TABANIDÆ, DESCRIPTION OF THE EARLY STAGES.

The Tabanidæ belong ecologically to the so called hydrophytic area, as has been pointed out by Osburn. On the whole, they may be called aquatic or semiaquatic, though not all of them pass their larval stage actually in the water; at the same time they show traces of an adaptation to plants which may serve to understand their phylogeny. The larvæ may be aquatic, semiaquatic, or even terrestrial in habitat, but always live more or less hidden and are seldom seen. They are, according to Malloch, "found rarely among decaying leaves or in low and somewhat marshy spots in fields." Neave and others, however, have obtained them in quantity from the muddy banks of rivers and brooks.

The eggs are laid in clusters of one or several layers, forming a compact flat or conical mass; they are rarely laid scattered, and never singly. The clusters may consist of several hundred eggs, the eggs being held together by a sticky substance. The whole mass is often covered with a dark shining varnish or with a chalky substance. These masses are deposited usually on plants or sticks at the edge of ponds, streams, and lakes, seldom on dry ground, and sometimes also on stones above water, but this is unusual.[2] The single egg is spindle-shaped or cylindric, and narrowed at the ends, white, yellow, or pale brownish when first laid, but later often pigmented, brown or shining black, the color being due to a pigmentation of the chorion. The period of incubation is short, from 3 to 9 days, but in some cases the hatching process is delayed for weeks by atmospheric conditions, while the embryo is fully developed and may be caused to hatch by artificial stimuli. All the eggs usually hatch at the same time, the young larvæ at first sticking together but soon losing their hold and tumbling down. In many cases only part of the eggs of one mass give birth to larvæ, about half of them being infected by small hymenopterous parasites.

[2] Macquart's statement that the female deposits its eggs in the ground is erroneous.

The newly hatched larvæ drop into the water or onto the ground, according to the egg-laying habits of the species, and little is known about them. The young larvæ resemble the full grown in appearance, but are generally more transparent, showing the internal organs, a pair of black eye-spots, and Graber's organ in its primary condition, which is later described. The chordotonal organs have been described in young tabanid larvæ. The nervous system is clearly visible. The young larvæ may be fed on tiny crustaceans, crushed insect larvæ, etc., but are not always successfully reared.

Of the young larvæ some have strongly developed tracheal trunks which enable them to float, while others sink to the bottom.

All tabanid larvæ are highly predacious, feeding on other insect larvæ, earthworms, and probably all animals they can get hold of, not sparing their own kind. Wahlberg found them, according to Brauer, semiparasitic in lepidopterous (noctuid) larvæ. Perris reports that he has found them under stones feeding on larvæ of *Rhizotrogus* and on *Melolontha* (imago?). According to Brauer, young larvæ burrow into other larvæ (of beetles) and leave these only when they completely fill the skin of the host. Brauer once obtained the larva of *Hæmatopota* from the larva of *Helops lanipes*, from which, as it seemed to be molting, the tabanid larva made its way. Walsh states that they feed on snails; Hine and others fed them on earthworms. Young larvæ can be fed on small crustaceans (King). It is not known what food they take in nature, and also whether they can subsist on vegetable food. Del Guercio's statements on this point are probably erroneous. Mitzmain and others have observed their cannabalistic tendency, at least in captivity. As Patton and Cragg have stated, the larvæ of the large species and of the nearly allied full grown stages of the smaller species feed almost exclusively on earthworms whose body juices they suck out; this explains why gregarine cysts (*Monocystis*) are not uncommonly found in the alimentary tract of the imago. Malloch says that the food of the species occurring in rivers is mostly tipulid and other larvæ which burrow in the soft banks of the rivers or occur in the river bottom or in drift.

Structure of the Mature Larva.—All the known larvæ of tabanids, with the exception of the somewhat aberrant *Gonoips*, are elongate,

cylindric, slender, tapering at both ends, capable of contraction and extension, their body consisting of a head and twelve segments, the last segment being very short. The larvæ are eucephalous, with head well developed but small, bearing the three-jointed antennæ, attached to the anterior angles of the head just above the palpi, the basal joint being short, the others of varying length. A bunch of stiff spines, either short or moderately long, is situated above each antenna and on each side of the labrum. All the essential mouthparts are present and have been studied in many species. They are well adapted for seizing the prey, and constitute a very formidable apparatus (Patton and Cragg). The essential organs are the mandibles and first maxillæ, and of these the former are the most powerful weapons. Each mandible is a stout rod of chitin, slightly expanded at the base, to which the muscles are attached, and narrowed distally to a blunt point; the rod is curved downwards and forward, and on its concave border has many coarse serrations. The maxillæ are similar in shape and general disposition, but are smaller and less heavily pigmented, and are more pointed. Both pairs of appendages can be thrust out from the head in a downwards and forward direction when the larva attacks its prey, by means of the protractor and retractor muscles attached to the base of the rods. One sometimes becomes aware of the existence of these organs when handling the larvæ, as they are used in defense as well as in attack, and are capable of inflicting a sharp nip, though they do not draw blood. The maxillary palpi are simple and two-jointed, the distal joint being much smaller than the proximal one. The dorsal and distal extremity of the head is projected forward as a short and fleshy labrum. A labium is likewise present. The head is attached firmly to the pharynx, and is retracted and exserted together with it. The pharynx itself is an elongate chamber of the usual type and is easily seen in the living larva. The eye-spots are placed on the dorsal side of the pharynx, not on the outer cuticle, and follow the movements of the pharynx. The pharynx leads posteriorly to the esophagus, which is narrow, but wider and more muscular than that of the adult. It is usual to find that the esophagus is wider and more muscular in insects whose food is solid or semisolid than in those which take only fluid food.

The head itself, as far as it can be exserted, is strongly chitinized and usually brownish in color, while the rest of the body is generally pale, whitish or grayish, but in many species marked with a regular dark pattern, which in others may be absent. Following the head are the three thoracic segments, of which the first is the shortest, and these are followed by nine abdominal segments, the last one forming the respiratory tube, and the next to the last segment bearing ventrally the anus on a fleshy prominence. The body surface is generally smooth, well chitinized, and half transparent. The fourth to tenth segments are provided at their anterior margin, or not far from it, with a strong circular fleshy ridge, containing circular muscles, and bearing the parapodia, eight in number when all are present, or fewer if, as may be the case, the dorsal ones are lacking. The ventral parapodia are usually better developed and placed somewhat closer together. The parapodia may be retracted and exserted, and, provided with many curved spines outwardly, play a part in locomotion. The ridges in some species are not very prominent, are usually covered with a fine pubescence rendering part of the body surface opaque, and are often pigmented dark, forming the patterns spoken of. The spinous processes of the prolegs pass through imperceptible gradations into the fine pubescence of the dull or pigmented areas.

The larva of *Goniops chrysocoma* differs in appearance from the other known tabanid larvæ by being apparently much shorter and thicker in the posterior half of the body than in the anterior where the segments are much tapered and considerably longer, but as the figures are based on specimens preserved in alcohol, we cannot pass a definite judgment on their systematic position.[3]

Integument.—The integument in general can be divided into more or less distinct dorsal, ventral, and lateral areas, which differ in their finer structure, all being either longitudinally striated or more or less smooth and shining. The lateral areas may be subdivided into an upper, middle, and lower portion, marked off from one another and slightly differing in structure. All these structural features of the surface of the integument are probably vestigial traces of formerly more marked characteristics, and are important in any effort to separate different species of larvæ.

[3] See also *Goniops* in general, p. 56.

The structure of the chitinous membrane has been studied in detail by Lécaillon in *Tabanus quatuornotatus* Meig. The membrane consists of three layers or zones, the innermost soft and of greatest thickness, the two outer ones hard and elastic; of the latter the inner (median) is much thicker than the external one. By staining methods the three layers can be differentiated. The fine striation of the cuticle is formed by the median layer, which is thickened in the form of narrow longitudinal ridges. The hairs are formed exclusively of the external layer. The muscles to the integument are attached to the median zone.

The muscular system is strongly developed, consisting of the retractor and extensor muscles of the head, and probably similar muscles governing the retraction and extension of the syphon; also of muscle fibers arranged more or less vertically to the integument, and circular muscles which function in the contraction of the circular segmental ridges and the retraction and exsertion of the parapodia. The muscular system is partly illustrated but not described by Graber (1882) (Plate 8, Fig. 100, *a*). Lécaillon (1906) notes the muscular insertions in the integument, stating that the muscles pass through the inner chitinous layer, losing here their transverse striation, and being inserted in the median layer. Various muscles go to the organ of Graber, described later, by means of which this organ is displaced in various directions. (Plate 9, Figs. 104 to 106, and Plate 10, Figs. 112, 115, and 116.)

Alimentary Canal (Plate 7, Fig. 98).—This has been studied by Patton and Cragg in *Tabanus albimedius* (also in other species?). It is much shorter and less complicated than that of the larva of *Stomoxys calcitrans*, described by the same authors. The esophagus opens into a short, cylindric proventriculus, which is also a highly muscular structure, and is sharply distinguished from the succeeding part of the gut by its clear translucent appearance in the fresh condition. The mid gut extends from the proventriculus to the hind end of the body, and is thrown into one or two simple coils, not constant in their position. It is separated from the proventriculus by a short constriction, and is again constricted at the posterior end, just anterior to the opening of the Malpighian tubes Between these points the lumen is wide and is thrown into numerous sacculations

by the contractions of the muscle fibers in the wall. The mid gut is, at least in the species studied by Patton and Cragg, of a striking orange-red color in the fresh condition, and is filled with a semisolid mass of a light chocolate color, which oozes out if the wall is punctured in dissecting. The hind gut is short and simple, and is coiled up in the posterior end of the abdomen. The total length of the gut is about twice the length of the body of the larva. The salivary glands are simple and tubular, and bear a remarkable resemblance to those of the adult insect.

Respiratory System.—The respiratory system resembles that of the mosquito larva. There are two large lateral tracheæ which run the whole length of the body on each side of, and slightly dorsal to the alimentary canal. According to Patton and Cragg (1913), these communicate with the external air through an opening, which can be closed, on a small prominence on the dorsal surface of the penultimate segment. This statement appears to be erroneous. Brauer (1883) states that the tabanid larvæ are usually metapneustic, the last segment with a vertical respiratory fissure or the last two segments forming a respiratory tube. According to Malloch (1917), they are always metapneustic. The respiratory tube is of varying length, sometimes very short, sometimes more slender and tube-like, or forming a short acute spine. The two main air-filled trunks are much inflated in some species, enabling the larvæ to float at the surface of the water, and narrow, almost filiform in other species. As they pass forward, they give off slender branches of tracheæ to the body cavity in each segment. In the anterior part of the body the tracheal stems, in the species where they are generally much inflated, become slender, describing one or several semicircular loops and tapering into fine tracheæ going to various organs. The tracheal branches going to the body wall of each segment may be homologous with those leading to the abdominal spiracles in amphipneustic larvæ.

Dorsal Blood Vessel.—The dorsal blood vessel is easily seen in transparent larvæ and ends blind, in the cases observed, before the eleventh segment.

Malpighian Tubes.—The Malpighian tubes are, to judge from Patton and Cragg's figures, four in number, in young larvæ irregularly crowded in the posterior part of the body between the two tracheal trunks, and of brownish, greenish, or yellow color.

Fat Body.—No observations of the fat body have been made, except that Graber (1878) describes it, in *Tabanus autumnalis* (?), as a network of pale lobes and trabeculæ with dendritic tracheal expansions, lying dorsally and above the new organ described by him.

Gonads.—These organs, which must be present in the larva, have not been noticed or described by any author.

Nervous System.—The nervous system of the larva consists, according to Graber, who studied the young larva of *Tabanus* (*Chrysops* ?), of a large upper and lower cephalic ganglion, and a chain of large ganglia arranged like beads and extending about half the body length. According to Graber, this would be the only instance of the nervous system having this structure (Plate 8, Fig. 99).

Sense Organs.—The eyes have been mentioned; their function is not clearly understood. Antennæ and palpi are probably olfactory. Tactile bristles occur on the body surface and have been studied by Graber who finds them connected with several ganglion cells (Plate 8, Fig. 100, *b*). Chordotonal organs, similar to those in *Corethra*, are found laterally in each segment near the surface of the integument, and are of the mono-, di-, and triscolop type (Graber), and are connected with the tracheæ. According to Graber, in small larvæ, the chitinous body walls themselves act as a tympanum, thus explaining the absence of any special tympanal apparatus (Plate 8, Fig. 100, *a*).

At the posterior end of the larva, situated dorsally and adjacent to the blind posterior end of the heart, on the eleventh segment, is an organ of unknown function, called Graber's organ, after its discoverer (1878). It consists of a pear-shaped sac, the broader end of which is anterior; the posterior end narrows down to a fine tubule which opens on the integument of the body between the last and the next to the last segments. Within this sac there is a series of capsules set one behind the other in the long axis, and within each of these capsules is a pair of small, black, pyriform bodies, each attached to the anterior side of the capsule by a delicate pedicel (Plate 9, Figs. 103 to 108, and Plate 10, Figs. 109 to 120). These bodies diminish in size from the anterior end, the first being considerably larger than the rest. They are easily seen through the integument of the living larva. The outer sac is an invagination of the integument, and as

such has a chitinous lining. Graber believed that the structure is an auditory organ, but Lécaillon supposed it to be a gland. Berlese, according to Patton and Cragg, does not consider that it has been proved to be a sense organ, though it is well supplied with nerves. Paoli has shown that the young larva has only two pedunculate bodies, and that with each successive molt a new capsule with two more such bodies is formed. Having observed that *Tabanus* larvæ are able to produce a slight crackling noise, Paoli believes the structure to be a sound-producing organ, but it is difficult to understand what ecological meaning such an organ could have.[4]

The pupæ of tabanids are classified as orthorhaphous (Brauer), opening in the act of hatching with a dorsal longitudinal slit. They strongly resemble lepidopterous pupæ (*Mumienpuppe*, pupa obtecta), having wing and leg cases firmly attached to the body and covered with it by a chitinous membrane. The pupal body is subcylindric, abruptly pointed or rounded anteriorly, and tapering somewhat posteriorly; it is generally yellowish brown to ferruginous brown, finely wrinkled, and has a lateral tuft of hairs on each abdominal segment. On either side of the head are the antennal sheaths, pointing outwards, and on each side of the median line two large tubercles, each with a central hair; below these are two raised areas with sharp edges, separated by a deep ridge. Lower still there is a pair of elevations, also with raised edges, and on the ventral surface of the head one or more tubercles. The segments of the thorax are indistinct; the mesothorax bears the large raised ear-shaped spiracles. The abdominal segments are free and about equal in length, and have one or more fringes of hairs near their hind margins; the second to the seventh segments inclusive have well marked lateral areas, covered with long hairs which are continued into the dorsal and ventral surfaces and correspond to the lateral areas in the larval integument. These hairs increase in length from before backwards, and are best developed on the seventh segment. The eighth segment is short, and is provided with six projecting spurs or teeth, and with a large anal tubercle. In the male the tubercle is ribbed and bounded anteriorly by a continuous fringe of strong spines; in the female the tubercle is smaller and the

[4] For details concerning the structure of this organ, refer to pp. 29–43.

fringe of spines is widely separated. The pupæ of many of the larger species of *Tabanus* have in addition a lateral tuft of spines situated on a ridge. Patton and Cragg were the first to notice structural differences in the male and female pupæ.

Brauer (1883) says that he classifies the tabanid larvæ with the Leptidæ and Acanthomeridæ (to which they are undoubtedly nearly related, as also to the Asilidæ) under *Homoeodactyla tanystoma*, of which the larval characteristics are:

> Larva meta- or amphipneustic or with tracheal gills, usually with eyes at the sides of the head capsule, the latter usually hidden in the following rings, generally more or less retractile, and behind it eleven or twelve body segments. Mandibles hook-like; extending out of or under them in their concavity are the maxillæ, which are soft-skinned, and the laterally prominent maxillary palpi; antennæ short. Labrum hooked or horn-like, exserted. Pupa a free "mummy-pupa."

Malloch (1917) proposes to unite the Tabanidæ with the Leptidæ to a superfamily Tabanoidea, having the following characteristics:

> "*Larva*.—Head small, wholly or partly retracted, permanently retracted portion with an arcuate dorsal plate over the longitudinal rods; mandibles stout, hook-like, curved downward; maxillæ well developed, wholly or largely membranous, the palpi well developed; antennæ distinct, pedunculate. Body cylindrical, with or without pseudopods; lateral abdominal spiracles absent in Tabanidæ, small, but no lateral spiracles distinguishable in Leptidæ; apical spiracles in a vertical fissure in Tabanidæ, exposed and separated in Leptidæ."
>
> "*Pupa*.—Head with strong cutting armature; antennæ with or without distinct annuli. Thoracic respiratory organs sessile. Wings and legs closely fused to each other and to thorax; fore tarsi overlying midpair, the latter overlying hind pair, the pairs successively longer, hind pair not extending beyond apices of wings. Abdomen with seven pairs of lateral spiracles; segments armed with transverse series of slender bristles which become progressively stronger from base to apex of abdomen."

Brauer's characterization of the Tabanidæ is the following:

> *Family Tabanidæ*.—Larva usually metapneustic. Eyes distant from the mouth-parts, situated laterally and back of the head capsule; the latter divided by a fissure at the inserted end, at the hind end open and prolonged backwards into long rods, hidden in the following segments. Mandibles hooked, often serrated at the border; labrum forming a hook-like projected septum between them. Body eleven-segmented, often girdled with retractile fleshy tubercles which are often developed as prolegs at the ventral side only. Last segment with a ver-

tical respiratory fissure, or the last two segments forming a respiratory tube. Pupa free, without crown of hooks at the fore end, antennal sheaths laterally exserted, parallel to the head. Between them four swellings or ridges, arranged in a curve and formed by chitinized folds. Above them three tubercles, forming a triangle (anlage of ocelli) and behind them outwards two larger tubercles. Beneath the wing sheaths on the inner margin of the sheath of the compound eyes on each side are two small tubercles lying one above the other. Anal segment with six divergent cone-shaped pointed hooks. Spiracles behind the head and on the seven abdominal segments distinct and large, the former with kidney-shaped or ear-shaped margin (*Therioplectes*) and often very large. The pupa rests in the ground or, in some species (*Therioplectes*), remains in the water.

Malloch describes the body of the larvæ as "circular in transverse section, elongate, tapering at both ends, and with encircling locomotor swellings at the segmental sutures in all genera except *Goniops*."

Malloch's description of the tabanid pupa, based on the study of a number of species, is the following:

"*Pupa*.—Head without projecting thorns. Thoracic respiratory organs sessile, connected subcutaneously with a large cavity on each side of median line close to anterior margin of prothorax. [Plate 13, Figs. 153 and 154.] Wings and legs rather short. Abdominal armature consisting of 1, or 2 closely contiguous, series of bristles on each dorsal segment except first, and a weaker transverse series on ventral segments; apical segment ending in six stout processes which are more or less radiate and pointed. [Plate 13, Figs. 162 and 163.]"

On the habits of the larvæ before pupation, facts have been published by Neave (1915).

Before the larvæ have reached their full growth, which in many cases signifies the beginning of a resting period, they usually lie buried in the mud, head downwards, with their syphons projecting immediately above the surface of the mud or of a shallow layer of water above it if it is present. In the resting stage the syphons do not seem to be used and the larvæ remain several inches below the surface for weeks or even months. This is presumably an adaptation connected with a climate in which there is a very marked dry season, and, consequently, a risk of the mud in which they are lying more or less drying up. Pupation appears to take place in normal circumstances an inch or more below the surface, though occasionally in captivity individuals pupated lying horizontally upon it. The pupa is normally upright in the mud, and after pupation, as soon

as the case has hardened, it works its way up by means of its rings of spines and the aster (a name proposed for the terminal whorl of spines), until the pupal head lies just below the surface, being often visible from above. The pupa at first is usually of a pale yellowish or greenish color, but darkens as the imago develops within, the process beginning with the eyes.

Neave observed that in normally fine weather the imagos of all species almost invariably emerged between noon and 3 p.m. They were more irregular during spells of dull and rainy weather. The process of emergence seems to be similar in individuals of all genera, whether *Chrysops*, *Hæmatopota*, or *Tabanus*. The head of the pupa splits in the median dorsal line and the imago rapidly emerges until only the end of the abdomen, which is at first enormously elongated, remains in the pupa case. The wings at this stage are milky white and the darker markings, if any, are barely visible. The imago usually remains in this position for two or three minutes before completely leaving the pupa case. It is capable of flight very soon after this, but if undisturbed sits for about half an hour on any suitable object near by while the wings dry and assume their normal coloration, and the abdomen its normal shape. During this period several drops of a milky white fluid (the meconium) are passed through the anus.

Bionomics.

On the bionomics of the tabanids in the early stages we possess many interesting notes by Neave (1915). From the results of a year's collection of adults in one locality, and from other evidence, it is probable that the majority of tabanids, at least of Nyasaland, have only one brood a year. This is certainly true of nearly all the species of *Tabanus* and of *Dorcalæmus fodiens* Aust. It is possible, however, that certain species of *Chrysops* and *Hæmatopota* may be double-brooded; much doubtless depends on the larval food supply, climate, etc.

According to Neave, in the case of many species the larva grows very slowly after hatching and often takes six months or more to become full grown. It then, especially in species of *Tabanus*, goes through a resting period, during which it remains buried in mud or sand, sometimes at a considerable depth. In contrast to the lengthy

larval stage, the pupal period is short, varying, in Neave's experience, from 10 to 16 or 18 days, according to the species and the climatic conditions, the longer period being usual for the larger species of *Tabanus*.

One difficulty connected with the question of these flies having more than one brood a year arises from the fact that even in larvæ from the same batch of eggs the rate of growth is extremely variable, and consequently the processes of pupation and emergence do not take place simultaneously in a certain proportion of the individuals. Some of the remainder take longer to reach maturity, others seem to pass through an extended dormant period. The adults arising from these emerge at irregular intervals, often months later. This probably explains the capture of odd specimens of any species long after the usual season. It would also seem not improbable that individuals which miss their normal season for pupation in some circumstances continue in the larval stage until the following year. Thus Neave possessed in his laboratory in January and February examples of larvæ of *Tabanus corax*, some still in a dormant state and others not yet mature, the season for the adult flies being over by the beginning of January. It would appear that these would not have produced imagos until the following December, though Neave was unable to decide this point on account of his return from Nyasaland to England. Hine states that the larvæ of *Tabanus stygius*—probably—hibernate twice before giving the adult.

Surcouf and Ricardo, however, assert that in spite of the opinions to the contrary the tabanids seem to have, at least in Algiers, often two generations in a year. These authors have taken from a horse several fresh specimens in May (1908), while the same species is taken in France and Algiers in the fall. Maxwell-Leffroy and Howlett state that at Pusa (Bengal) there are apparently three broods of *Tabanus* yearly, flies emerging at the beginning and end of the hot weather (about February and June) and at the end of the rains (October). These statements should be compared with those made by Neave. Hibernation takes place in the larval state in India in all species which were observed by Maxwell-Leffroy and Howlett; and the same holds good for all known American species (Hine), larvæ being found late in the fall and again in early spring. No pupæ have been found in the winter.

Special Anatomy of Tabanid Larvæ. Graber's Organ.

Almost the only detail of the structure of tabanid larvæ which has been studied more extensively is a peculiar organ situated on the dorsal side of the segment before the last, and discovered first by Graber (1878), who, while conceding that it has very little resemblance to an auditory organ, nevertheless may have to be placed in this category. As the situation of the organ is rather unusual if it is auditory, Graber recalls the discovery by Grobben of similarly situated organs in the larva of another fly, *Ptychoptera contaminata* (Tipulidæ), and of which the auditory function seems fairly certain. Whether similar organs are found in other dipterous larvæ is not known; Malloch (1917) does not mention them. The organs seem to occur in all tabanid larvæ, but to be more easily seen in the young stages; they have been overlooked by some later investigators, notably Hart (1895) and Hine (1901–06), but have been seen and figured by Walton (1908) for *Goniops chrysocoma* (only as dots), and by Mitzmain (1913) for *Tabanus striatus*, all of whom have apparently overlooked the European literature on the subject, Mitzmain assuming that the organ must either be peculiar to the larvæ observed by him, or have been overlooked by the previous authors.

Graber found the organs in the abdomen of the larva, at the sides of the two last segments. They consist of a cornea-like inflation of the integument which is covered inside with its epithelium. The open inner side of this vesicular invagination seems to be closed by a membrane supported by radial elastic fibers, the fundus of which can be extended by means of a strong muscle. According to Graber, the presence of a special nerve, which, however, approaches the vesicle without any specialized termination, is proof that we are dealing with a sense organ.

However, what characterizes them as auditory organs are two or three otolith-like bodies found in the liquid contents of the capsule. These abdominal otocysts consequently differ from antennal structures described elsewhere by Graber, first, by belonging to the integument itself, second, by their inner wall not being formed by chitin but by the epithelium. As a consequence of this, the development of cuticular auditory hairs is naturally also suppressed, and a new type of

auditory capsules is developed, which Graber distinguishes as otocysts without cilia (aciliate) from other known otocysts (ciliate otocysts).

The organs were found by Graber (Spring, 1878) on a dipterous larva collected with other material, on the bottom of a mud pond in a brick factory. The larva (Plate 9, Fig. 103) was when extended about 40 mm. long, cylindric, strongly spindle-shaped, and pointed at the end, and except the dark intestinal system, of an almost glassy transparence. Eleven segments were counted, which with the exception of the terminal ones, were surrounded by a girdle of papilliform retractile processes. Unfortunately, as literature was not accessible, the species could not be determined.

In order to see the organ in the fresh condition, the larva is laid on its ventral side and fastened with an elastic holder. The organ (Plate 9, Figs. 104 to 108) lies in the median line of the dorsal side, behind the termination of the dorsal blood vessel, and immediately behind the border incision between the ninth and tenth segments. By focusing from above, at first the body cuticle becomes visible, which derives from the presence of longitudinal ridges the extraordinary elasticity characteristic of these larvæ. When focusing farther down, the epithelium is seen, consisting of flat polyhedric cells, and following this a network of pale lobes and trabeculæ with dendritic tracheal expansion; namely, the fat body. Immediately underneath these tissues lies the organ in question, which is consequently completely separated from the integument. It consists of a pale capsule 0.3 mm. in length and pear-shaped, its free and broader end directed upwards, while its pointed hind end is prolonged into a narrow tube. The capsule being of considerable size is visible even at slight magnification. Moreover, it is not likely to be overlooked because of the intensely black bodies included in it, which strongly contrast with the light background.

In detail the structure of the organ appears to be peculiar. From the capsule proper with its tube the nerves and muscles attached to its anterior end may be distinguished. The whole capsule together with the terminal tube appears, as Graber says, to be a cecum-like invagination of the ectoderm. Unfortunately the origin of the terminal tube could not be found by Graber; it apparently lies in the

last body segment and is in no way connected with the intestine, which terminates at the end of the segment before the last, or with the sexual organs. In Graber's account it remains questionable whether the terminal tube, as was supposed, really originates in the integument. The tube and the capsule forming its enlarged terminal part behave histologically like a glandular formation. The main layer is an epithelium consisting of large cells. This is seen best, especially after treatment with 35 per cent potassium hydroxide, at the distal (head) end of the capsule. The cells appear as elongated, sac-like, pale compartments, separated from one another by bridge-like septa, always showing a dark nucleus accompanied by a small nucleolus. In the remaining part no distinct cell borders can be distinguished, but only large granulated nuclei. In the terminal tube the latter are arranged alternating behind one another, similarly as in the narrow secretory ducts of true glands. The epithelial tube is covered at the outside by a thin homogeneous covering which may be considered as a tunica propria.

The structures belonging to the third stratum, that is, to the chitinous membrane, are peculiar. Leaving aside certain complications, we find first a chitinous capsule corresponding to the epithelial one, which is prolonged into a narrow canal corresponding to the terminal tube. This canal pursues, inside the terminal tube, an undulating course, curved alternately to the right and left, suggesting the muscle in the stem of a *Vorticella*.

The chitinous capsule enclosed within the epithelial vesicle is often very thin and delicate compared with the thickness of its matrix, and is perfectly transparent. Its free (inner) surface, however, shows at high magnification small tegula-like overlapping scales. In this chitinous capsule the black bodies already mentioned are enclosed.

At low magnification these bodies appear as simple homogeneous globules. At the highest magnification, however (Plate 9, Fig. 107), they are found to be hollow chitinous structures with a somewhat wrinkled surface, which after the fashion of a volumetric flask are prolonged into a narrow hollow stem or peduncle. The bodies remain perfectly black and opaque even when boiled in caustic potash; consequently they seem to have very thick and strong chitinous

walls. Sometimes it seems as if they are filled with a dark tough substance, projecting in the shape of a papilla or of a granulated string into the lumen of the peduncle. Eight of these pedunculate bodies, as Graber calls them, were present in the larva examined, arranged in four pairs, lying one behind the other, giving to the vesicle the appearance of an internally segmented organ. The bodies of the first two pairs are of about the same size, 30 μ in diameter, the length of the peduncle 26 μ, its width at the tip 1.8 μ. The bodies of the two following pairs immediately touching one another are considerably smaller than the rest, their diameter being only 20 μ.

The compound nature of the organ appears more distinctly than in the serial arrangement of the pedunculate bodies, in the repetition of the covers in which they are enclosed. There are not four such covers or secondary sacs, however, but only three, as the two posterior pairs of pedunculate bodies are contained in a common envelope. The first sac is formed by the capsule itself; that is, by its rounded (head) end. The pedunculate bodies are observed hanging by their pedicel from the slightly invaginated upper wall, and, like the following ones, turned from the inside towards the outside in a somewhat oblique direction. The space in which the bodies are found, however, is separated also from the remaining lumen of the capsule, and, as far as could be seen, by a transverse septum issuing from the side walls.

The second pair of pedunculate bodies is, however, surrounded by an independent sac entirely separated from the main capsule, the fixation of the pedunculate bodies themselves at the inner upper wall of the secondary vesicle being exactly the same as that in the first capsule. This second sac, however, is not completely closed behind the pedunculate bodies, but is forming here only a neck-like contraction, its walls otherwise being continued into the following sac.

The third inner sac resembles the second one in all essentials, but encloses, as already mentioned, two pairs of pedunculate bodies, which again, in a manner analogous to that in the former cases, are inserted into its walls.

Probably the last two sacs are evaginations of the fundus of the common capsule, fitted into one another like the sheaths of an onion. In this way the first pair is surrounded by one envelope, the second

by two, and the third and fourth by four envelopes. The accessory parts of the organ have also been studied by Graber.

At a moderate magnification it is possible to discern, in addition to the tube extending backwards from the capsule, two other ligaments originating at the sides of the pear-shaped part, so that the whole organ appears to be held in place by three ligaments, one of which is the terminal tube. The two lateral ligaments extend obliquely, crossing the two large tracheal trunks, in a forward and outwards direction, inserted in a place not determined by Graber but probably lying in the seventh segment. At higher magnification the anterior ligaments are found to be muscles, and immediately behind the place of origin of these muscles two pairs of nerves are seen attached to the capsule. The first nerve being rather thin forms immediately at the head of the capsule a thick ganglion-like swelling, in which several pale nuclei are discernible. The character of the ending of this nerve has not been determined; Graber assumes that it enters into connection with the capsular epithelium. Nothing more is known about the termination of the second nerve which is much thicker than the first one.

All the parts described, the capsule, the terminal tube, the muscles, and nerves are connected with one another and with the surrounding organs, as also with the tracheæ and with the band-like extensions of the heart muscles, by a peculiar connective tissue. The latter shows the greatest affinity with certain elastic reticulate tissues of higher animals. These tissues play a part, according to Graber, in the extraordinary changes in the relative situation of the parts during the movements of the larvæ. Graber discusses the possible function of the organ he described, assuming that it must be either a gland or sense organ. The presence of a duct leading to the exterior seems to indicate the former, but the arrangement of the contents seems to contradict this view.

If the organ is considered a gland, this would imply that a secretion formed by the epithelial cells is discharged into the chitinous capsule. In this case, however, no explanation is found for the presence of the pedunculate bodies, not to mention the various interior secondary capsules. The pedunculate bodies have no free opening, and while they are hollow and possibly have been formed by invagination of

the walls of the capsule, the place of invagination seems to have disappeared.

Graber therefore decided that the organ must be a sense organ, which, however, according to its peculiar structure and position cannot be either an organ of touch, smell, taste, or sight, and is consequently placed among the auditory organs. Graber also believed that he found the two main features of a cystoid auditory organ, namely a cyst-like cell system, connected with a nerve, and an internal fluid medium which may carry the sound. There were lacking only the characteristic elastic appendages of the auditory cells.

Graber assumes that under certain conditions the auditory cells may be stimulated by waves of sound even in the absence of special end organs, in the same way that the light-perceiving elements of the eye in part are also affected by light-waves in the absence of special terminal organs as found in highly developed organs of sight.

The presence of the peculiar pedunculate bodies seems, more than anything else, to have determined Graber's view, in as far as they form a good analogy to the otoliths commonly found in auditory organs of the cyst-like type. The pedunculate bodies are comparatively heavy, thick-walled bodies and are attached to the cysts by means of thin and probably elastic filaments or strings; they may be compared with the clappers of a bell, which facilitate their function as otoliths.

Graber classifies all auditory organs occurring among insects, as (1) elementary organs, consisting of isolated hearing cells or auditory hairs, (2) cystoid auditory organs, gymnotocysts, and chitinotocysts, found in crustaceans and many insects, and (3) tympanal organs, provided with auditory rods as found in *Orthoptera*. The chordotonal organs are related to the latter and differ by the absence of a tympanal membrane. The organ in question is classified among the cystoid organs.

Henneguy found the same organs in small larvæ resembling larvæ of *Stratiomys* (Lécaillon), (Plate 10, Fig. 109), which undoubtedly belong to *Tabanus*. The organ in this instance (Plate 10, Fig. 110) is different. It comprises a cellular mass situated in front of the organ and evidently connected with the dorsal vessel, a capsule following it, containing two pigmented bodies arranged as described by Graber, and, finally, a cyst prolonged backwards into a filament but

not containing pigmented bodies. Henneguy is inclined to place the organ among the chordotonal organs.

Lécaillon, having in 1904 sent some larvæ of *Tabanus quatuornotatus* to Henneguy, studied the organ at the latter's suggestion. He records a number of facts which may facilitate further research on the subject. The principal results of his observations are as follows:

1. Graber's organ exists in all larvæ of *Tabanus quatuornotatus*. It should consequently be sought for among the tabanids, and possibly also in related families.

2. It already exists in the larvæ hatching from eggs, but it continues to develop as the larvæ grow.

3. In very young larvæ its structure corresponds to that described by Henneguy; in fact he made his observations on very young larvæ.

4. As the larvæ grow, the primitive structure becomes more complicated and more closely resembles the structure described by Graber. This author observed full grown larvæ.

5. Consequently it can be stated that Henneguy's description applies to the young condition of the organ, and that of Graber to the fully developed condition.

6. In Lécaillon's observations, as shown by the figures, the pigmented bodies do not always remain regularly arranged in pairs, contrary to Graber's description. They are seen frequently placed one behind the other in the posterior duct of the organs. Sometimes there is an odd number of them in this duct, which means undoubtedly that these granules can sometimes be discharged or expelled.

7. While nothing can be said with certainty about the function of Graber's organ, Lécaillon considers it glandular in nature rather than a sense organ.

The figure of the larva given by Lécaillon (Plate 10, Fig. 117) was obtained from a living larva of *Tabanus quatuornotatus* one month old, seen in dorsal aspect with light falling through, a larva at this stage being still very small. Two other figures (Plate 10, Figs. 118 and 119) give the aboral extremity of two other larvæ slightly older than the first one.

As seen in these figures, the main part of the organ of Graber occupies the median and dorsal region of the next to the last segment, in almost its entire length, and is situated not far from the two large tracheal trunks, which open, one near the other, at the end of the last abdominal segment. It consists mainly of an oval sac or cyst prolonged posteriorly into a tube. The oval and dilated part is subdivided by a septum in two cysts placed one behind the other. Careful

study of this region shows that the two cysts are not completely separated and, on the contrary, communicate with one another in their axial region; in other words, the septum which separates them is incomplete. The tubular region which prolongs the posterior cyst begins towards the middle of the segment next to the last and opens in the median line not far from the openings of the two tracheal tubes. This tubular region is, consequently, about as long as the dilated portion of the organ. The fundus of the sac, that is its anterior region, has thicker walls than any other part. There is in this region a cellular mass similar to that described by Henneguy in the organ of the undetermined larva which he examined.

The ink-black bodies can be found, according to Lécaillon, in both capsules and in the tubular portion. The first capsule always contains two which are situated anteriorly, one to the right, the other to the left of the median line. By carefully examining their arrangement it is found that they are attached to the cellular mass at the fundus of the cyst, each by a small pedicel, as has been described by Graber and by Henneguy, whose observations are confirmed by Lécaillon. These pedicels do not show on Lécaillon's figures, which, as he says, were drawn from living larvæ under conditions when the details were not clearly visible.[5] The second capsule contains sometimes also two of the black bodies, arranged as in the first capsule, but often it does not contain any of them.

The tubular region often contains no black bodies. But frequently and especially in older larvæ, it almost always contains a varying number. The maximum number which Lécaillon found was six, but there may be only five, four, three, or two, even in larvæ of the same age and living under the same conditions. As the caliber of the tubular region is narrow, the black globules are placed here in a lengthwise row, and not in pairs placed transversely, as in the capsules.

As Lécaillon wished to ascertain whether the black bodies could eventually be expelled to the exterior, a larva which had six globules in the tubular portion of the organ was placed under observation. Eight days afterwards Lécaillon found that the tube contained only

[5] I found that living tabanid larvæ up to 15 mm. in size can be examined by means of a compound microscope, without suffering injury from the pressure necessary to hold them in place.

two globules, four evidently having been expelled. At about the same time another larva of the same age was found to contain only the two bodies of anterior cyst; all the others had been thrown out.[6] In summarizing Lécaillon states:

(1) The black bodies are formed by the cellular mass which constitutes the fundus of the capsule. (2) The globules are detached periodically to be expelled to the exterior after a longer or shorter time. (3) They are regenerated at the bottom of the cyst when the others have been expelled.

These conclusions of Lécaillon appear only partly warranted. From observations made hitherto there is no indication of a relation of the discharge of these bodies and their new formation.

Concerning the nature of the black bodies, Lécaillon states that they are not hard as if they were mineral granulations; they are not affected by reagents, as acid fixation fluids; they may be cut when the organ is sectioned. Consequently they seem to be a pigmented material, excreted by the cellular mass which forms the bottom of the cyst. Lécaillon alludes, in suppport of this view, to the fact that the larvæ of *Tabanus quatuornotatus* have a completely white body though the substances found in the digestive tract (organic relics) are usually blackish. The pigment derived from the alimentary substances, or formed normally in the body, might be excreted by means of this particular gland.

Paoli in Florence in 1907 studied the same organ in various tabanid larvæ which, on the authority of Bezzi, are said to belong probably to *Tabanus cordiger* Meig. or *Tabanus autumnalis* L. Paoli, however, asserts that in all probability many species of this family possess Graber's organ more or less developed and also with slight modifications in details of structure, according to age and species. For the description of the larvæ studied by Paoli see pages 91–93, *Tabanus autumnalis*.

According to Paoli's description, Graber's organ is found in the visceral cavity, situated dorsally under the subcutaneous muscular strata, in the anterior third of the eighth abdominal segment between the two large tracheæ (Plate 10, Figs. 115 and 116). It is a little pear-

[6] I have been able to confirm Lécaillon's observations in the larvæ of *Tabanus atratus* (1916).

shaped cyst with a slight constriction in its wider part; its pointed end is turned hindwards. Inside this cyst are found, in the cases examined, several pairs of black rounded chitinous pedunculate bodies; each pair of these bodies is in turn enclosed in a special chitinous capsule filled with liquid. The attachment of the muscles and nerves to this cyst are described in detail. The cyst is continued, at its pointed end, into a contorted canal which opens in the deepest place of the infolding between the eighth and ninth abdominal segments (Plate 10, Fig. 115). The canal and the cyst are covered inside with chitin and completely surrounded by the hypoderma, so as to warrant the conclusion that the whole organ must be an invagination of the integument, as Graber had supposed, though he was unable to see the opening of the terminal tube.

According to Graber, the first cyst contains in its interior four pairs of pedunculate chitinized bodies; the first pair being in a closed capsule, the second inside another capsule, the latter not completely closed posteriorly, and finally the remaining two pairs contained in a common sac, open posteriorly.

Paoli states that the number of pedunculate bodies is not limited to four pairs, but varies according to the age of the larva. Graber also said (in his later work), that in the young larva there is only one pair of such pedunculate bodies, and that the second pair is formed later. Henneguy, who examined a newly hatched larva, found only one pair.

Paoli found four pairs in larvæ about 1 cm. in length, but in specimens of larvæ in later stages of development he always found a greater number, up to seven pairs in larvæ of almost 3 cm. in length. Each pair was found enclosed in a capsule which remains more or less open posteriorly, but which is always present; the largest of them, that is, that which is located anteriorly, has the thickest walls and is formed last, and it is only this one which is really living and in function, while all the others are dead and have no function whatever, differing little from one another, especially the capsules enclosing the smallest; namely, the oldest pairs of pedunculate bodies.

Each of the pedunculate bodies has the shape of a slightly elliptic ball, their surface being smooth or sometimes rugose. They are strongly chitinized, black, frail, and breakable, as under pressure

they may be reduced to small fragments; the pedicel is inserted obliquely, equal to the latter in length. The pedicel itself is chitinous but not black; at most it is brown in the neighborhood of its insertion in the globule; at the other end it has a widening by means of which it fastens itself in a little cavity of the wall of the chitinous capsule. The pairs of pedunculate bodies are attached each one to the central portion of the anterior wall of the capsule itself, and the pedicels are a little divergent so that the two spherical bodies are located a certain distance from one another, suspended in the liquid which fills the cavity of the capsule. The pedunculate bodies are of dimensions increasing in the direction of the head; in a larva with four pairs these bodies measured for each pair 11, 15, 16.5, and 18 μ in diameter.

Paoli explains the probable manner in which these cysts and the tube are formed. It has been stated that the latter opens into the innermost part of the sulcus which limits the eighth abdominal segment from the ninth; Graber did not see this opening but supposed that it was to be found in the last —ninth —segment and certainly independent of the alimentary canal and of the genital organs.

The origin of this organ is thought to be a sac-like invagination of the integument (Plate 10, Fig. 113). The most external part forming the terminal tube, the more internal one a cystiform enlargement, from the bottom of which the two pedunculate bodies originate, the latter consequently being nothing but modified hairs derived from the hypoderm surrounding the capsule.

In this way it is evident that in the young larva which has not yet undergone any molt there will be a primitive organ with only one pair of pedunculate bodies. At each successive molt the chitinous stratum of the organ remains in place, involved by a new stratum formed around it by secretion from the hypoderm, and the old capsule is pushed back distally. The new layer consequently forms a new cyst with a transverse division separating two halves in the anterior of which two new pedunculate bodies are formed, this being the new organ, while the posterior half consists of all that existed before. In this manner, with the successive molts, an increase in the number of the cysts and of the pedunculate bodies is brought about; the capsule wall which is thinner when it belongs to a younger stage;

is no more easily visible in the final form. This hypothesis is, according to Paoli, the only one which explains satisfactorily the manner in which this strange organ is formed, and it is sufficiently supported by observation. In fact, the whole organ is covered by the hypoderm, the latter not being very different from that which covers the body integument internally.

In a preparation of a whole larva which had molted recently Graber's organ was made up of five pairs of black pedunculate bodies, and one pair, anteriorly located, had the pedunculate bodies already formed but entirely colorless; this capsule was evidently recently formed and its pedunculate bodies were not yet completely chitinized and fully colored.

The further fact, that the number of pairs of pedunculate bodies increases with the age of the larva, clearly demonstrates that they are formed successively, and inasmuch as we have to deal with an organ dependent on the integument, these successive formations must have something to do with the molts as modifications of the integument. All the cells and nuclei described by Graber as surrounding the cyst and the tube consequently are merely cells and nuclei of the hypoderm which surrounds the whole organ.

Paoli discusses further the muscles and ligaments observed by Graber. It is found that the muscles as well as the nerves are attached to the anterior capsule, which alone forms the living and functioning part of the organ, while the remaining part is to be considered dead, having functioned in previous stages of development.

While Graber described only one pair of muscles, Paoli observed four pairs and stated that probably Graber's second pair of nerves corresponds to the second pair of muscles. Two large anterior muscles are attached to the anterior part of the capsule with a large surface of insertion extending from the dorsal posterior side of the capsule upwards almost to contact with one another. These muscles are those seen by Graber who assumed that they were attached with the other end in the fifth abdominal segment, the seventh as he erroneously assumed. According to Paoli, they are shorter and inserted in the sulcus between the sixth and seventh abdominal segments; they diverge anteriorly passing across the two large tracheal trunks. By the contraction of these muscles the organ is displaced towards the head.

The second pair of muscles corresponds probably to the elements which Graber assumed to be a second pair of nerves; their diameter is about the same as in those of the first pair; they are attached at the sides of the posterior parts of the cyst, extending backwards almost parallel to one another, and finally are found to be inserted at the sides of the opening of the terminal tube, in the furrow between the eighth and ninth abdominal segments. The action of these muscles consequently is directly opposed to that of the first pair, and displaces the organ posteriorly.

The two pairs of muscles described are the most important and are attached at the lateral walls of the cyst; the two following pairs are attached to the posterior border of the ventral side of the cyst. Of these, the third pair, more exteriorly located, is formed by two divergent muscles which are inserted at the sides of the ventral body wall, somewhat behind the sulcate prominence bearing the anus. The two muscles of the fourth pair are also attached to the ventral body wall, near the median line, in front of the lip-like hook-bearing expansion. Hence it appears that these two pairs of muscles pull the organ in the ventral direction, between the two large principal tracheæ.

Membranous ligaments are also described by Paoli. In addition to the four pairs of muscles and the terminal tube, the organ is held in position by membranes, the largest of which has the shape of a conical cap involving the capsule anteriorly and attached to the posterior extremity of the dorsal blood vessel, which terminates not far from the limit between the seventh and eighth abdominal segment. Other membranes, one on each side, envelop the basis of the muscles of the second pair, but Paoli was not able to find the insertion of their free end.

Concerning the nerves belonging to the organ, Graber claimed that it was richly innervated, describing two large nerves which formed ganglion-like masses near the capsule. These nerves, however, are muscles, according to Paoli, which are slightly inflated at their base. It is to be noted, however, that the ganglion-like swellings were reported by Graber not for the large nerves, but for the first thin pair. Paoli found two nerves which anastomose and fuse to a short tract, further on redividing into two branches, one more

slender which goes to the base of the muscles of the second pair, one larger which, extending below the muscles themselves to the anterior part of the capsule, corresponding to the pedunculi, while it is not possible to affirm with certainty that it has a relation with these. Paoli seems not to attach any importance to the behavior of this nerve.

All the muscles, in addition, receive in their course the terminations of other nerves. Those of the first pair, not far from their place of insertion in the cyst, receive two branches derived from a nerve which bifurcates in the neighborhood of the muscle itself, in fact so near that it gives the appearance that the nerves go to the cyst.

All the nerves and muscles described are related to the head capsule which encloses the first pair of pedunculate bodies. The remaining section, which contains the dead parts of the organ, receives towards its middle the endings of a slender nerve which, while it is a single one in the region of the hypoderm, branches into a large number of thin ramifications.

Concerning the function of Graber's organ, Paoli likewise dismisses the hypothesis of a glandular function, there being no indication whatever of the presence of glandular cells.

That the organ should have an auditory function is also thought to be of little probability, especially as it would differ from other cystoid auditory organs, by having its inner surface covered with chitin, bearing no hairs, but instead of them, pedunculate bodies. Otoliths are, moreover, practically never found among insects, and the organ in question would be unique also in this respect. Furthermore, in this organ, there seems to be no special preponderance of nerves as one would suppose to be the case in a sense organ, but rather a preponderance of muscular apparatus, in as far as numerous long muscles are connected with it in various directions, these muscles being richly innervated. The organ is consequently, according to Paoli, destined chiefly to be set in motion or to be extended in various directions; in fact, displacements were observed in anterior, posterior, and vertical directions, and also lateral displacement seems to be possible if the muscles acted on one side alone.

Paoli, in seeking for the function of this organ, recalls his own observation of the larvæ producing a crackling noise, under water as well as in the air, similar to the sound produced by small electric discharges. He thinks that these sounds might be produced by means of this organ. By the action of the muscles the cyst would be subjected to deformations by means of which the pedunculate bodies would be caused to hit against one another. The elastic membranes also would aid in this process as well as the chitinous walls of the capsule. The two large tracheæ would serve as resonators.

The objection that such an organ of sound should lie at the surface of the body rather than in its interior, is met with the consideration that these larvæ are aquatic, living below the surface of the water or in the mud, where the possession of an organ producing sound in the air would be of little value. The song of the Cicada becomes much feebler when the insect is submerged under water, but the larva in question produces its sound as well under the water as in the air. As the pedunculate bodies are enclosed in a liquid, the conduction of the sound would also occur easily in a liquid element. If the peculiar sound produced by the larva was not caused by the action of this organ, there would be, according to Paoli, no other organ by which it could possibly be produced. Paoli has consequently decided to assign to the organ a sound-producing function.[7]

[7] I have in the meantime found occasion to observe tabanid larvæ, and was eager to test Paoli's observations. In fact the crackling noise was freely produced by full grown *Tabanus atratus* larvæ, and also, in harmony with Paoli's statement, it was chiefly heard when the larvæ were disturbed and defending themselves with their sharp mandibles. The coincidence of the two phenomena was so close that I am bound to assume that the sound is produced by means of the mandibles. As is well known, the mandibles of tabanid larvæ are strongly chitinized and provided with a serrate inner edge. Very near this and slightly below, the maxillæ, also chitinous, are situated. In the act of biting, the mandibles are suddenly exserted with considerable force and it is conceivable that in this act their serrate edge strikes the maxillæ, producing a sharp sound. The action itself may have some physiological importance, in as far as it serves in the laceration of the skin or body wall of animals attacked, while the sound produced appears to me more accidental, as, for instance, the gnashing of teeth in carnivora.

Hence it is not likely that Graber's organ has the function ascribed to it by Paoli. Also Paoli's opposition to Graber's view is not well founded in as far as he himself assumes that the pedunculate bodies are modified hairs. They could

CHRYSOPS (MEIGEN), EARLY STAGES IN GENERAL.

In Fabricius' *Systema Entomologiæ* and *Entomologia Systematica* I have found no statement of the early stages of any species of this genus.

Zetterstedt says that, according to Fabricius, the larvæ live in the ground. However, Zetterstedt himself saw a large number of apparently new emerged adults at the borders of a lake, which would indicate an aquatic habitat.

According to Hart, the larvæ and pupæ of *Chrysops*, as well as the imago, are distinguishable from those of *Tabanus* by the antennal structure. Otherwise the *Chrysops* larvæ closely resemble in structure small or young *Tabanus* larvæ. The dull pubescent annuli are partly present in *Chrysops*, but the longitudinal lateral lines, except on the prothorax, are shining and almost entirely without pubescence. There is very little pubescence here, however, in some young *Tabanus* larvæ. The species described by Hart (*Chrysops vittatus* Wied.) is easily recognized by the dark patch on the last segment.

The distinguishing characteristics given for larvæ of *Chrysops* compared with those of *Tabanus* are the following: "Last antennal joint much longer than the one preceding, dorsal areas of thorax striated like those of abdomen." Neave compared the syphon of the

consequently take the place of the auditory hairs which are missing, and as the pedunculate bodies, which in reality are modified hairs, would assume the function of otoliths, there would be no exception to the general rule that true otoliths are not found among insects. Functionally the organ could well belong to the group where it was placed by Graber. Also the fact remains that, even according to Paoli, nerves go to the fundus of the cyst, where the pedunculate bodies are attached. The problem cannot be regarded as solved by Paoli's hypothesis. On the other hand, to assume a true auditory function seems equally hazardous; it seems more probable that the organ might play a part in the senses of equilibrium and orientation, which, however, can only be determined by further investigation.

Chrysops larva with that of *Hæmatopota* (Plate 5, Figs. 73 and 74), and found the syphon much more elongated in *Chrysops*.

Beling (1882) is the first to describe the pupa of a *Chrysops*, which had been found at the edge of a brook. A *Chrysops* larva which pupated became known first through Hart (1895). This larva remains, until recently, the only one described. After Hart, our knowledge is increased chiefly by Hine, especially with reference to oviposition and egg stage, on which Patton and Cragg have also made observations. The larvæ and pupæ of several African species became known through Neave's work (1915).

The little *Chrysops* pupæ have longer antennæ, and the thoracic spiracular prominence is more nearly in a vertical plane than in *Tabanus*, its inner edge being more strongly elevated. The lower free edge is crossed by sharp folds, making it serrated. In *Chrysops* the abdominal spiracles are subcylindric near the apex; the spinose fringes consist of long teeth only; and the terminal teeth are long and rather narrow at the base.

The distinguishing characteristics of the pupæ are the following:

"Antennæ surpassing adjacent margin of head; fringes of abdomen of long spines only; inner margin of thoracic spiracular prominences sharply elevated, lower margin serrate-edged; abdominal spiracles slender, subcylindrical near apex; size small."

The eggs of *Chrysops* are deposited, as we learn from Hart, "in one flat tier, forming an oval or diamond-shaped area, pointed at one or both ends." We know, however, from Hine's observation on *Chrysops celer*, that there are exceptions to this rule and there are species of *Chrysops* which oviposit in several layers after the fashion of *Tabanus*. Most of the eggs of *Chrysops* are black, according to Hine, and are placed in a single layer, but there are exceptions to this, for the eggs of *Chrysops celer* are never darker in color than brown, and are placed in at least three layers one upon the other.

With regard to the habits of the pupal stage, Hine's observations are of interest, as he saw around fresh water ponds myriads of pupa skins of *Chrysops* with just the anterior end projecting above the surface of the ground.

The following are the notes which we possess on various species of *Chrysops*, arranged alphabetically.

Chrysops bimaculosa Neave.—An African species, allied to *Chrysops centurionis* Aust., discovered by Neave (1915) on Mt. Mlanje, in southern Nyasaland. A typical male and female and one other of each sex were bred in October and November, 1913; one female was collected in November, 1912.

Of the four individuals above mentioned three were bred from collected pupæ and the fourth from a larva which resembled that of *Chrysops longicornis*, though considerably larger, with a somewhat less strongly pigmented anal segment and with well marked hairs on the syphon.

The hooks of the pupal aster, especially the upper and middle pairs, are decidedly elongate (Plate 13, Fig. 167, *a*, *b*, *c*).

Imago, larvæ, and pupæ had been taken on the banks of a wooded stream.

Chrysops callidus Osten Sacken.—A common and widely distributed species, recorded from most of the eastern states, reaching Florida in the South, Indiana in the West, but apparently not occurring in northern New England. Hine has described the oviposition of this species, indeed has watched the entire process of oviposition, which usually occupies from 20 minutes to half an hour, during which time something like one to three hundred eggs are laid.

The female alights on the leaf head downwards and begins to push the tip of the abdomen forward towards the sternum of the thorax, placing the protruding end of an egg against the leaf. This end sticks fast and she then moves the tip of the abdomen backwards until normal position is reached and the egg is free. By the same movement one or two eggs are then placed to one side of this one and two or three on the other side of it. The unfinished end is soon observed to be V-shaped, the female moving gradually forward and placing the end of the abdomen to one side of the V and depositing eggs along down until the apex is reached, then changing the tip of the abdomen to the outer part of the other side of the V and placing eggs along it down to the apex on this side.

This process is kept up, the female changing regularly to the outer part of the opposite side of the V each time the apex is reached. Between 9 o'clock and noon seems to be the favorite time of day for

oviposition, as with other species of *Chrysops* and *Tabanus;* Hine has seldom observed females ovipositing at other hours of the day.

The eggs when first laid are clear white but gradually get darker until they become permanently dark brown or black. It took eggs of *Chrysops callidus* 5 or 6 days to hatch, and it required about a day longer in the case where eggs were kept in the shade the whole time than in cases where the eggs were in the sun during the day.[8]

The writer has made some additional observations on *Chrysops callidus*, which are published elsewhere.

Chrysops celer Osten Sacken.—A species recorded from Maine, Massachusetts, Pennsylvania, New Jersey, Ohio, and North Carolina. This species is common in Ohio during the latter half of May. The eggs have been observed commonly by Hine along the margin of ponds and artificial lakes, clinging to various kinds of foliage overhanging the water. The female has been observed ovipositing on different occasions, and is the only species of the genus placing its eggs in masses composed of layers one above the other as in *Tabanus*.

Chrysops dispar Fabricius.—This species is widely distributed in India, Ceylon, Malay, and adjacent parts. We possess some information on its early stages through the work of Patton and Cragg in Madras (1913).

[8] In the summer of 1916, I collected the eggs of *Chrysops callidus* O. S., in Princeton, N. J., from which the young larvæ were obtained. These are half transparent, whitish, and their structure is analogous to that of most tabanid larvæ, differing, however, from the young larvæ of *Tabanus*, as far as known to me, by having the main tracheal trunks not inflated, but of about equal diameter all along their course. This character ("not provided with air sacs") is, however, given by Patton and Cragg for all "small tabanids" observed in Madras, including species of *Chrysops*, *Tabanus*, and *Hæmatopota*. It causes the larva of *Chrysops callidus* to sink to the bottom instead of floating to the surface. The larvæ of this species molt soon after hatching, and can live under water for a considerable time, but as all the larvæ died in young stages, I am unable to say what their further habits are, and whether they spend most of their life in the water or later on invade the mud of the shore. At a pond where large numbers of *Chrysops* had oviposited the previous summer, in spring no larvæ could be detected in the mud of the margin, while other tabanid larvæ were numerous.

For further details see Marchand, *J. N. Y. Entomol. Soc.*, 1917, xxv, 149.

Chrysops dispar lays its eggs, as do all the small species of tabanids observed by these authors, invariably on blades of grass just at the edge of a shallow stream, or on the leaves of the lotus plant at the edges of small ponds, but never over deep water.

In Madras the smaller species of tabanids always lay their eggs in the afternoon, commencing about 4 p.m. *Chrysops dispar* has been seen ovipositing as late as 7 p.m. An egg mass of this species, on a blade of grass, and also a single egg, is figured by these authors (Plate 1, Figs. 14 and 15), but no description of the larva is given.

The larvæ of the smaller species of tabanids (including *Chrysops dispar*) contain no air sacs, according to these authors, and if they fall into deep water they die. It is important to recognize this in breeding experiments and to place them in trays with only a little water.

Patton and Cragg's text-book contains notes on smaller species of tabanids some of which may apply to this species, but are not clearly referred to it.

Chrysops indus Osten Sacken.—This species is recorded from New Jersey, New York, Canada, and Ohio. According to Hine, it appears in Ohio usually by the middle of May. It is the first species to appear in the spring and females have been observed ovipositing on plants growing along the margin of a small lake on the University grounds in Columbus, Ohio. The eggs are placed in single layers on grass blades that hang over the edge of the water.

Chrysops longicornis Macquart.—This is an African species, the early stages of which have become known through the work of Neave (1915). The species is the most abundant of the genus in the neighborhood of Mt. Mlanje, southern Nyasaland, where Neave's investigations were carried on. The flies prefer well wooded localities and Neave thinks it probable that all stages exist throughout the year.

The larvæ were first discovered at the end of August, 1913, and to Neave's surprise, in view of the habits of the adults, were found in the mud of a small marsh and stream bed in an open spot with only comparatively thin woodland near it. Many other examples were subsequently taken, both in similar places and in less unexpected spots on the banks of wooded streams, etc. Except for an occasional

freshly emerged individual, the adult flies were not taken in these open places and appear therefore to migrate from them after emergence and to return to them for the purpose of oviposition. If this is the case, it is another example of the possibilities of error in searching for the breeding place of a species in the spot most frequented by the adults.

The larvæ, figured by Neave but not described, were obtained in considerable numbers from September on, a few being still obtainable even in January and February. In the figure (Plate 5, Fig. 72) the eleventh segment shows a broad dark pigmented band, dorsally reaching somewhat anteriorly to the middle of the segment, ventrally including the anal prominence, while the tube-shaped posterior third of the segment appears to be free from pigment, as also the slender tube-like twelfth segment (syphon). The circular ridges show only traces of pigmentation. A good illustration of the pupa is given (Plate 11, Fig. 130), which, however, shows no peculiar characteristics by which it could be differentiated from a *Tabanus* pupa. The pupal asters of male and female pupæ are shown and differ considerably (Plate 13, Fig. 171, *a*, *b*).

Chrysops magnifica, *var. inornata*, Austen.—An African species, according to Neave, not rare in the Mlanje district of southern Nyasaland, during the rains from October to April.

This species was bred from the larva by Neave, but the larva so closely resembles that of *Chrysops longicornis* Macq. that Neave never succeeded in separating it satisfactorily, since in both species the usually distinctive characters of the pigmented anal segment and syphon were variable.

The pupal aster (Plate 13, Fig. 166, *a*, *b*) resembles that of *Chrysops longicornis*, but the middle pair of hooks is stouter and somewhat more curved, and the shape differs somewhat, especially in the male. The pupal asters of the male and female are illustrated.

Chrysops mæchus Osten Sacken.—A species recorded from New Jersey, District of Columbia, Illinois, Ohio, and Kentucky. Hine has observed the females ovipositing on foliage overhanging a mill race at Georgesville, Ohio, June 4, 1899, but does not describe the eggs.

Chrysops mœrens Walker (synonym *æstuans* van der Wulp).—Recorded from Illinois, North Dakota, Wisconsin, and Ohio. This is the species in which oviposition was first observed by Hart (1895), who saw these flies from August 3 to 10 flying among the marginal rushes of Fourth Lake, Sand Lake, and Slough Lake in Lake County, Illinois, and ovipositing on the stems of the rushes.

The egg is described by Hart as follows:

"*Egg.*—[Plate 1, Fig. 2, egg masses.] Length 1.6 mm., diameter 0.25 mm., cylindrical with rounded ends, straight or slightly curved, smooth, slightly opaque, cream color when laid, becoming dark fuscous brown, placed in a single flat layer, obliquely stacked as in *Tabanus*, about one fourth of the length of each egg being visible at the surface, the remaining three fourths being covered by those stacked against it. The mass is about 10 mm. long and 3 or 4 mm. wide, its outline variable, usually diamond-shaped, both ends pointed, or at one end short or truncate, making it more or less triangular."

Eggs of this species were observed by Hine (1906) at Sandusky, Ohio, during the first days of July and were present in varying numbers during the following two months.

While the female is ovipositing she is not easily disturbed; consequently one has an excellent opportunity to watch the procedure. An illustration given by Hine (Plate 1, Fig. 1) was made from a photograph of a living specimen which was found in the act of egg laying and was carried, with the leaf, to the laboratory where the picture was taken. "During the whole time," says Hine, "she continued ovipositing without showing any signs that she was aware of what was going on or that she had any concern for the welfare of her eggs."

The method of placing the eggs is similar to that recorded for *Chrysops callidus*.

The female alights on the leaf with her head downwards and begins the process by pushing the tip of the abdomen forward towards the under part of the thorax and placing the protruding end of an egg against the leaf. The end sticks fast in consequence of the glue-like substance which accompanies it, and she then moves the tip of the abdomen back to its normal position, thus freeing the egg. By similar movements one or two eggs are placed on one side of the first, and two or three on the other side of it. The unfinished end soon becomes V-shaped; she moves slowly forward and lifts the tip of the abdomen to one side of the V

and places eggs along it downwards until the apex is reached; then changes to the other side of the V and places eggs along it, downwards to the apex on this side. It was noted in specimens of this species that sometimes a female would place as many as three rows of eggs on one side, one after the other, before changing to the opposite side. It is only necessary to study a mass of these eggs in order to see the precision with which the different specimens are arranged.

The eggs are placed on various aquatic plants, often standing in rather deep water and at times as much as 20 rods from shore. Hine always found them on scattering plants around the edges of grassy areas and not back among the dense growth; consequently they are easily seen, not only on account of their conspicuous location, but also because of their shining black color, which contrasts strongly with the green leaves to which they are attached.

Their coloration renders the egg clusters conspicuous, and Hine suggests that hand picking might be of consequence in the control of *Chrysops*. In order to demonstrate what could be done in the way of gathering eggs of this species, Hine went out on the morning of July 17, in Ohio, in a small rowboat, and collected for an hour. At the end of this time a count showed 433 masses, and an average of 250 specimens to each mass, a result obtained by counting several and striking the average, giving a total of 108,250 single eggs taken as a result of the hour's work.

Eggs of *Chrysops mœrens*, laid from 8.45 to 9.30 a.m. on July 13, hatched before noon of July 19, thus making the incubation period 6 days. This is the shortest incubation period Hine has observed for any species of Tabanidæ.

Of the larvæ, Hine says that after hatching they drop into the water, and he states that, in the natural breeding grounds of the flies, it is almost impossible to find the very small larvæ after they have dropped from the eggs and have become more or less scattered among the debris which is usually plentiful in these places.

In order to ascertain whether the young larvæ can be killed by spreading a film of kerosene on the surface of stagnant water over which eggs are placed, Hine (1906) performed some experiments. A tank of water was used, on the surface of which half a pint of kerosene was placed to each square yard of surface. *Sparganium* leaves to which the eggs were attached were brought in from the marsh and

put into a bottle as one would arrange a bouquet, and this was placed on the bottom of the tank so that the parts of the leaves to which the eggs were attached were a foot or more above the surface of the water which contained the layer of kerosene. Even under these conditions an exact count could not be obtained, because the kerosene appeared to affect different specimens differently. Some were killed very quickly, some died after an hour or more, while others did not appear to suffer particular inconvenience from the treatment. Further observation is necessary to be able to give conclusive statements regarding the matter.

Chrysops relictus Meigen.—A European species, and the first species of the genus in which reliable data were obtained, by Beling (1876), on the pupal stage. From three pupæ found on July 16, 1876, in the sand of the border of a small meadow brook, two imagos were produced on July 24 and 25. The third did not develop.

Beling's description of the pupa is as follows:

Pupa.—12 mm. in length, 3 mm. in diameter, of dirty brownish yellow color. Head shining, strongly brownish anteriorly; lower frontal margin with four broad, rounded teeth in a transverse row; above these teeth two small tubercles, each with two stiff brown moderately long hairs; further down posteriorly two similar tubercles separated by a larger distance and each bearing only one such hair. Dorsally, at the border between the head and thorax, are two brownish ear-shaped longitudinal ridges diverging posteriorly. Antennal sheaths laterally appressed to the head, short, terminating in a point, not much marked. Abdomen nine-segmented, brown, with blackish segmental incisions, less shining than the head and the leg and wing cases. First abdominal segment very short, deeply emarginated in the middle of the anterior margin; third to eighth abdominal segments inclusive dorsally near the posterior margin with a transverse row of densely placed backwardly appressed, pale bristle-like teeth of unequal length, gradually becoming longer on the posterior segments and extending also over the ventral surface of the segments. Anal segment ending in six claw-like spines arranged divergently, of which the two upper ones are slightly smaller than the remaining four.

Chrysops vittatus Wiedemann.—A common species distributed all over the eastern United States as far west as Kansas and Iowa. This is the first species of *Chrysops* of which the larva has been described (Hart, 1895).[9] The larvæ were found, according to Hart, in

[9] Recently, in the spring of 1917, I have found larvæ of this species from which a male imago was reared.

connection with those of Bittacomorpha, Limnophila, and Sialis, in the weedy, swampy little stream at Station I of his entomological survey of the Illinois river valley. They were quite common here, occurring in the mud and the mats of dead stems, rarely floating at the surface. The first were seen March 28, but they continued to occur up to April 15, increasing slightly in size. In the breeding cage they burrowed in the mud and through the vegetation.

In the latter part of May the water was allowed to dry up and on the 28th all that remained was poured off. From June 1 to 3 three pupæ were formed in the damp mass of dead vegetable matter resting on the mud in the cage. Two imagos emerged June 9, both males, the third failing to transform.

The coloration of the larva readily distinguishes it from other known tabanid larvæ. Hart's description of larva and pupa is quoted below.

"*Larva.*—[Plate 3, Fig. 39.][10] Length 10–15 mm., diameter 1.6 mm. Head light colored, mouth parts pale, tips of maxillary palpi in line with end of labrum; body whitish, a mottled appearance within, at the middle of the body."

"Dorsal and ventral areas striate, striæ entire, distinct, and not very fine, the lateral striation a little finer, that of the prothorax very fine, with a small smooth spot adjoining the smoother surface of its ventral area; the latter shorter than the dorsal, not including the anterior pair of setæ; median sulcus scarcely dull-pubescent. Meso- and metathorax with lateral impressed lines, and dull-pubescent pale annuli, but the lateral lines almost without pubescence. Fleshy false feet of abdominals rather prominent, dorsal pair united into one, there being no narrowing near the median line; annuli very pale except on the last two or three segments; last segment white basally, the remainder covered with dull blackish microscopic pubescence reaching forwards to the anal prominence, a triangular extension each side of the middle above, often a small spot accompanying each; respiratory tube whitish, spine sometimes projecting."

"Tracheal trunks sinuate posteriorly, crossing and recrossing in front of the middle."

"*Pupa.*—[Plate 13, Figs. 154 and 157.] Length 9–10 mm., diameter 2 mm. Light brownish, ferruginous, obsoletely transversely wrinkled, head and thorax shining, abdomen duller."

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[10] In the quotations, as well as in the rest of the paper, the figure numbers refer to the illustrations in the present monograph.

"Antennal sheaths not very thick at base, surpassing the marginal angulation above them; carinated tubercles not prominent, lateral notches broad and shallow, palpal sheaths indefinite, rather distant; setiferous tubercles scarcely darker; ocellar tubercles replaced by pale dots. Rima of thoracic spiracles (Plate 13, Fig. 157) strongly elevated from inner side, so that the flat top of the prominence is nearly vertical, the upper edge of the rimal border forming a sharp carina and its anterior extremity ending at the tip of the marginal extension in an acute angle; the free lower edge is crossed by sharp ridges, giving it a serrate profile; rima less curved at middle, more strongly at each end, scarcely hooked; inner notch with radiating striations."

"The abdominal fringes consist of a single row of pale spines on each segment, rather long except dorsally on the second, where they are shorter and thicker. The abdominal spiracular tubercles arise from a slight elevation, tapering from a comparatively small base as far as middle, thence nearly cylindrical to apex, which bears a subcircular rima; on the anterior slope a small transverse groove, not longer than the rima; tubercle about as high as its basal diameter. Last segment with six nearly equal terminal teeth, their points marking the angles of a hexagon; slender, even constricted at base, twice as long as their diameter near base. Lateral spines almost wanting; ventral fringe in front of anal tubercle in male; a tuft of about five spines on each side in place of this fringe in the female."

Chrysops wellmani Austen.—An African species, which, according to Neave, occurs in fair numbers near Mt. Mlanje, southern Nyasaland, from September to January.

The larvæ of this species (Plate 3, Fig. 50), differ strikingly from any of the other *Chrysops* larvæ seen by Neave, in their strong pigmentation. In the figure it is seen that while segments 1 to 3 are free from pigmentation, segments 4 to 11 show the regular tabanid pigmentation on the transverse circular ridges, leaving an area around the prolegs free from pigment. There are no longitudinal stripes, but on segment 11 there are two subdorsal dark spots (in addition to the broad posterior band), traces of which are also visible in the figure of the syphon of *Chrysops longicornis*.

The larvæ were obtained in the beds of forested streams with those of *Chrysops longicornis* and *Chrysops magnifica, var. inornata*, but were much less common. They were found only between the middle of October and the end of November. There is considerable difference in the hooks of the aster in the two sexes of this species, the upper and lower hooks, especially the former, being much reduced

in the female. The pupal asters of both sexes are shown (Plate 13, Fig. 168, *a*, *b*).[11]

Dorcalœmus fodiens Austen.—An African species, on which some observations have been made by Neave (1915) in southern Nyasaland.

Some examples of what may be the larvæ of this species were found, though the point could not be decided, as Neave was obliged to leave before they reached maturity. They were captured during December, January, and February in some swampy ground on which a patch of maize was growing. These larvæ were of fair size, some 30 mm. in length; the syphon was very short and had a distinct pigmented ring on the anal segment resembling that in *Hœmatopota* larvæ. In the more mature specimens traces of intersegmental pigment were present.

At the time these larvæ were captured no other larvæ of so large a species were obtainable, and as *Dorcalœmus fodiens* is the only large tabanid which is on the wing in March and April, there are grounds for thinking that the larvæ belonged to that species.

Gastroxides ater Saunders.—A black species of a genus allied to *Chrysops*, with elongate slender legs and long antennæ, occurring in Pusa, Bengal, India (Maxwell-Leffroy and Howlett, 1909). The larva is said to breed in hollow trees. No more details are available. A picture of the adult is given by Maxwell-Leffroy and Howlett.

[11] In the summer of 1916 I found eggs of another species of *Chrysops* which differed considerably in appearance from the two types described hitherto. These eggs were found on the under surface of leaves of the yellow pond lily (*Nuphar*), four or five clusters in all, and one on a *Pontederia* leaf. The eggs were white, and placed vertically on the surface of the leaf, one beside the other, the cluster being roundish when seen from above, presenting an even dull white surface formed by the tips of the eggs, and with clean-cut vertical outer walls. After a few hours these egg clusters turned somewhat brownish, but never became very dark. In one case the female fly was observed ovipositing, but escaped before being captured. It appeared to belong to *Chrysops univittatus* or possibly *Chrysops lugens*. The larvæ hatched within 5 days, molted soon after hatching, and did not differ appreciably from those of *Chrysops callidus*.

For further details see Marchand, *J. N. Y. Entomol. Soc.*, 1917, xxv, 149.

GONIOPS (ALDRICH), EARLY STAGES IN GENERAL.

The following brief characteristics of the early stages of *Goniops* are given by Malloch (1917).

"*Larva.*—Mandibles stout, slightly curved, apically truncated; antennæ elongate, 3-jointed, basal joint stout, tapering apically, about twice as long as apical 2 combined; apical joint much shorter than preapical; maxillary palpi 2-jointed, the apical joint slender and distinctly shorter than the basal. Thoracic segments very distinctly tapered anteriorly, abdomen stout, roughly oval in outline, the whole body appearing pyriform or slightly club-shaped; abdominal segments with rather irregularly arranged transverse series of locomotor tubercles; spiracular chamber in form of a vertical slit."

"*Pupa.*—Head without projecting thorns; antennal sheath short, curved downward. Prothorax about one-third as long as mesothorax; wings short, extending to apex of first ventral abdominal segment; apices of hind tarsi slightly surpassing apices of wings. Armature of dorsal abdominal segments consisting of stout thorns in a transverse series, 2 of which, near middle of segments 2 to 7, are much stronger than the others; later the series are discontinued some distance from margins; apical segment with three strong thorns on each side, between which are several weaker protuberances."

The eggs are, as stated by Malloch (after Walton (?), McAtee (?)), usually deposited on the under side of leaves of various plants, and when the larvæ hatch they drop to the ground, living afterwards among the decaying leaves and other vegetable debris. They are probably predacious like other Tabanidæ.

The egg masses are parasitized by a proctotrypid parasite.

McAtee briefly compares the larva and pupa of *Goniops*, apparently the only native pangoniid genus of which these stages have been described, with the comparatively well studied immature forms of the true tabanids, such as *Tabanus* and *Chrysops*.

Hart says (quoted by McAtee):

"All the larvæ of Tabanidæ studied agree in the following general characters: Body tapering at both ends, which are somewhat pointed; skin shining and glassy, with opaque markings of a microscopic felted pubescence. The palpi have short thick joints; the basal joint of the antennæ is quite short, and there is a bunch of stiff diverging recurved hairs between each antenna and the median line above."

The full grown *Goniops* larva, on the other hand, is pyriform and not at all pointed at the ends. Its skin, except on the head and prothorax, is not shining, but everywhere opaque and wrinkled or tuberculate. The palpi have long slender joints; the basal joint of the antenna is very long, much exceeding the two terminal joints, and the hairs on the antennal flap are flexible and attached to the surface of the head. The double second joint of the antenna of first stage larvæ is noteworthy.

Of the tabanid pupa Hart says:

"The mesothorax is one-half longer than the prothorax and the second to seventh abdominal segments are encircled by continuous fringes of slender spines."

In *Goniops* the pupal mesothorax is three times longer than the prothorax, and the fringes of spines on the abdominal segments are not continuous but interrupted and definitely grouped.

Goniops chrysocoma Osten Sacken.—A North American species, known from not very frequent captures, from New York, New Jersey, Pennsylvania, Ohio, and Maryland. Taken in June and July, usually on foliage.

Goniops chrysocoma (Plate 2, Fig. 28) is the only representative of the Pangoniinæ of which we know anything of the early stages, but in this species our knowledge is rather complete owing to the observations of Walton and McAtee.

Walton was the first to observe oviposition accurately and to describe the egg. However, Walton relates that the first fly seen by him was presented to him by Mr. Warren S. Fisher, of Highspire, Pa., who took a female in the act of ovipositing on a leaf of what proved to be *Angelica* on July 4, 1907, near Highspire. The plant overhung a small more or less permanent ditch of water, and it was naturally inferred that the larva might be aquatic in habit, in common with others of the family.

However, on June 14, 1908, while collecting on a dry hillside, in a brush patch, about five miles to the eastward of the former locality, Walton found another female of this species in the act of oviposition on the under side of one of the leaves of a small oak sapling, to which his attention had been attracted by a peculiar buzzing sound. The

female made no effort to escape, indeed it required considerable force to remove the insect from her position near the eggs. The immediate locality was a hillside pasture lot, half covered with scrub oak and berry bushes, dotted with clumps of false indigo. The nearest water was a small overgrown ditch some 60 feet distant.

On June 18 Walton visited the same spot near Highspire, with the hope of securing additional data, and was rewarded by finding another fly in a similar position on a leaf of the wild cherry, thirty feet distant from the water.

The eggs (Plate 2, Figs. 28 to 32), "which are deposited upon the under surfaces of the leaves of various herbaceous plants and trees" (Walton), in a three-sided pyramidal heap, are described by him as "yellowish white in color, about 1.5 mm. in length, slender, slightly curved, and resembling those of many other flies in general appearance." One of the heaps contained 534 eggs by actual count (Plate 2, Fig. 29).

The eggs of *Goniops* have been further observed, according to McAtee, during each of the years 1908, 1909, and 1910, on Plummer's Island, Maryland. On June 26, 1908, Mr. H. S. Barber collected a female and a large greenish white egg mass, which was laid on the under side of an oak leaf about 8 feet above the ground. The larvæ hatched June 28.

In 1910 McAtee found four egg masses on July 3 and two on July 10. One of the first four egg masses was collected. The larvæ hatched July 7. Another had been deserted by the female by July 10. The outer layers of eggs were black, and from these issued, on July 11, numerous proctotrupids, which Mr. J. C. Crawford says are *Telenomus*, probably an undescribed species. The two remaining egg masses of the lot found July 3 were covered by the females until July 10 (Plate 2, Fig. 28), a period of a week, during which time many eggs were added. These eggs, and the two masses discovered on July 10 as well (Plate 2, Figs. 30 and 31), were hatched by July 17. They were deposited on the under sides of *Eupatorium*, *Benzoin*, and *Hamamelis* leaves. Some of the empty egg cases (Plate 2, Fig. 32, McAtee), usually clung to the leaf after hatching, but in one instance not the slightest trace remained of an egg mass on a

witch hazel leaf. A fly heard by Mr. E. A. Schwarz giving its peculiar buzz on July 13, and which undoubtedly was ovipositing then, was located by McAtee on July 17. On July 24 the female was absent and the eggs were hatching. The larvæ, dropping to the ground, immediately burrowed in.

These observations show that the female *Goniops* guards the egg mass sometimes for a week at least, a fact which has escaped Walton's attention; that this precaution does not always prevent parasitism; that the period of incubation varies; and that the larvæ are fitted for a subterranean life upon which they enter as soon as hatched.

Walton placed the two batches of eggs found by him in breeding jars and on the evening of July 25 the first larvæ made their appearance. The second lot appeared two days later. The eggs are, according to him, yellowish white when deposited and change but little if any in color before hatching. The process of hatching was observed. The larvæ were quite lively when hatched and "it was a curious sight to see them come tumbling out of the eggs by dozens when the cluster was brought under the bright light." According to him, the period of incubation is from 7 to 10 days.

According to McAtee, eggs have hatched in from 2 to 11 days from the date of collection. But from the fact that eggs are added to the mass for several days, and that all hatch at the same time, it must be inferred that the eggs within the body of the female keep pace in development with those laid. To determine the true period of incubation observations must cover the process from the laying of the first egg to hatching (McAtee).

All the egg masses found on Plummer's Island in 1910 (McAtee) were on the steep north slope of the principal elevation of the island, which is a well shaded, cool, and damp locality. The finding of seven egg masses in this area of less than an acre in one season shows that *Goniops chrysocoma* is not uncommon locally, even though little is known of it and recorded captures are not numerous (McAtee).

As to the larval stage, I have already related Walton's observations on the hatching process. The larvæ obtained by him were divided into three lots, the first placed in earth entirely submerged in water, the second in dampened sand without food of any kind, the

third in a jar of damp earth together with some small angleworms. Twelve hours later the first lot were all dead; both of the other lots were lively and apparently in good condition. The second group continued to live without food for about ten days and then died. The remaining group lived for some weeks but finally died also, the angle worms being alive and uninjured.

Walton concludes that the larva is terrestrial (as confirmed by McAtee) and the period of incubation is from seven to ten days (see above).

The young larva (Plate 7, Fig. 93, *a*, *b*, *c*) is described as follows:

"The freshly hatched larva is slightly more than 2 mm. in length, slender, but capable of contracting the body into an almost spherical mass; in color it is pale yellowish white, semitranslucent. The head, which is capable of being entirely withdrawn into the first thoracic segment, bears several pairs of antenna-like appendages and an obtusely pointed chitinous hook."

"On each side of the median line of the body, within the second thoracic segment, there is a distinct pinkish spot; also on the last segment there is a pair of round black spots resembling stigmata [Graber's organ (?)]; elsewhere the body seems to be absolutely devoid of hairs or tubercles."

McAtee's description of the larva goes much more into detail. He also described the adult larva from a specimen collected by Mr. Theodore Pergande, and the pupa collected by him. In fact the greater part of McAtee's article is devoted to the description of the newly hatched and full grown larva and pupa of this species. In the description of the larvæ the segments are numbered from the head backwards. McAtee does not accept the customary use of the terms pro-, meso-, and metathorax for the three anterior segments of the larvæ, but speaks of them as the first, second, and third body segments, which they really are. In the newly hatched larvæ they are scarcely differentiated from the following segments. In the full grown larvæ, while distinguishable by the surface markings, their exterior features are homologous with those of more posterior body rings. In comparing and describing them, therefore, it is more natural to use numerical designations. McAtee's description of the young larva follows:

"*First Stage Larvæ.*—The average length of first stage larvæ of *Goniops chrysocoma* which have been preserved in alcohol is about 1 mm. In life they are

about twice as long. The larvæ are not tuberculate, but the margins of each segment from the third to the tenth, especially the front margins, are more or less raised into low rounded rings. On a larva with arched body definite transverse impressions behind the anterior fleshy annulus of each segment are apparent under magnification. They render the annuli conspicuous enough, in fact, to give an impression as of false feet to the naked eye observing the larvæ crawling."

"The mouth parts are exceedingly minute and hard to observe. In arrangement they suggest those of the full grown larva, described below, and the homologies have been made out accordingly, and, it is hoped, successfully. The drawing [Plate 7, Fig. 95, *a, b*] is strictly diagrammatic and is made up from a number of studies of larval heads, none of which showed all the parts in the position used in the drawing."

"Labrum (*lbr.*) short, pointed, black-tipped, and slightly curved downward. Labium triangular, not bifid as in full-grown larvæ. Maxillæ (*mx.*) fleshy, truncate-conical, with a short downwardly projecting lobe on the inner side of the distal end; palpus (*mx.p.*) arising from the end of the maxilla, first joint long, somewhat enlarged distally, tipped by a number of short rods or spines, one of which is larger and blunt. It may be considered a second palpal joint surrounded at the base by a group of spines. Mandibles (*md.*) fleshy, blunt-tipped, crenulate on lower edge, lying just inside of maxillæ. Antennæ (*ant.*) straight, tapering, directed forward; basal joint as long as first palpal joint, somewhat expanded distally, second joint double, one of its divisions longer and apparently tipped with a seta."

"First segment of body slightly inflated, first and second segments convex above, flattened beneath, lower lateral edge rather prominent. Second and third segments with two or three longitudinal furrows on each side. Second segment with two conspicuous well separated elongate brownish spots visible (apparently somewhat under the surface) on the dorsal aspect. Hind margins of segments becoming more undulate posteriorly, markedly so on ninth and tenth segments. Last segment with two round black spots, (spiracles) Graber's organ (?), set close together on the median dorsal surface; this segment with two more prominent ventral tubercles, two similar lateral ones, and other minute tubercles "

Both Walton and McAtee have seen the organ of Graber in the young larva of *Goniops;* the former speaking of a resemblance to spiracles, while the latter seems frankly to believe that he is dealing with spiracles. For a possible derivation of this organ from spiracles we possess, however, no evidence, except that in tabanid larvæ all spiracles except the terminal ones have disappeared.

Full Grown Larva (Plate 7, Figs. 92, *a, b, c,* and 94).—The full grown larva described by McAtee is one of two collected by Theodore Pergande, near Cabin John Bridge, Maryland, April 13, 1899. They

were found under stones covering the opening of mouse burrows. The color in life was gray. The general color of the preserved specimens was dark brown, the head black. The total length, when the head was retracted, is given as 17 mm., the greatest diameter 7.5 mm. With the head fully drawn out the larva measures 21 mm. For the details McAtee's description is quoted:

"Head convex above, flattened beneath, its lower lateral edge a well marked ridge made by the stretching of the skin over the prolonged basal supports of mouth parts. Anterior part of head marked off from posterior part by a band of very thin wrinkled skin; anterior fold of this band beginning dorsally just in front of two large lateral smooth areas containing the indefinite bluish white eye spots; fold descending obliquely over side of the head, ending ventrally between bases of maxillæ. Anterior part of head and areas surrounding eye spots, with glassy surface, remainder covered with thin wrinkled skin."

McAtee describes the mouth-parts in detail (Plate 7, Fig. 95, *a*, *b*.)

"Epistoma and labrum (*lbr.*) forming a thin lance-like projection between upper parts of oral apparatus. Upper edge of labrum grooved from opposite middle of antennal flap (*anf.*) to base of the mandibles (*md.*); provided with an unequally two-lobed caruncle just back of the upturned tip (*lbr.*). Higher lobe of caruncle and tip of labrum each bearing a solitary anteriorly directed seta [see Fig. 95, *a*]. Lower edge of labrum applied to labium (*lb.*); latter flat; thin lateral strips diverging from a distinct ramus behind being chitinized, the remainder flexible. Labium ending in a pair of rounded conical fleshy lips; flexible portions closely set with short yellow hairs."

"Mandibles black, claw-like, blunt, sheathed at base by lobes of maxillæ (*mx.*). The latter thin, flexible, following the curve of the mandibles; their slightly forward curved tips surpassing mandibles; their lower edge and inner side provided with numerous yellow hairs."

"Palpi (*mxp.*) arising from external basal flaps of maxillæ. First palpal joint inwardly and downwardly curved, second setiform, slightly curved downward and overlapping mandible.'

"Arising in the arch between epistoma and side of the head is a flap (*anf.*) which seems to be part of the antennal apparatus. It follows edge of epistoma nearly to base of mandibles, and curving down is attached to the posterior third of first antennal joint. From this point clear around the curve to where it parallels the epistoma the flap is fringed with long yellow hairs. First joint of antenna (*ant.*) slightly incurved (thus being directed outward), nearly three times as long as the two terminal joints together. Second joint conical, tapering gradually, directed forward and downward; third setiform and directed downward.'

"Anterior part of first segment very finely tesselated, the granules being arranged in irregular longitudinal rows. The head retracts into the posterior part of this segment, whose exterior is a longitudinally striate thin membrane inflated to gibbous barrel shape [Plate 7, Fig. 92]. The line of separation between the parts of this segment is marked by a ring of fine fleshy crenulations. Second and third segments surrounded about the middle by undulate crinkly thin-edged folds [Plate 7, Fig. 92, *a*, *b*, *c*] with five symmetrically placed backward angulations in each fold on each side. Parts of these two segments posterior to the folds with as many longitudinal sulci as there are angles in the fold, and longitudinally striate with fine irregular wavy ridges. Anterior third of second segment finely striate lengthwise. Part of second segment just in front of fold and anterior portion of third segment granular."

"On the fourth and following segments these folds are broken up into series of fleshy tubercles, on both sides of which the surface of the segments is raised into low ridges, which on the anterior segments have a few, and on the posterior segments several, low protuberances. The fourth and following segments more prominently ridged transversely on the dorsal area and longitudinally on the ventral area. A trace of longitudinal striation remains on posterior of three ridges (on each segment just described) or protuberances representing it. The fleshy teeth derived from the medium segmental folds largest and most numerous on the middle of lateral area of each segment, where they are heaped up into irregular elevations, with two more prominent points forming a series alongside; these elevations marked off by deep impressions both above and below and becoming more prominent posteriorly. There are three tubercles above (supralateral series) lateral prominences and about five below (infralateral series) on each segment behind the fourth.

"The fleshy fold is continuous across the dorsal area of fourth segment [Plate 7, Fig. 92]; back of this, dorsum of each segment marked by a depressed comparatively smooth elliptic area. These areas bounded in front by a varying number of thin fleshy teeth and posteriorly by a series of low broad longitudinally striate protuberances. Two of the latter fall into a series down the median line of the segments, on the ninth and tenth of which they become closely approximate, much more prominent, and round pointed. The first of the series (supralateral) of these tubercles above the prominent lateral elevations bounds the dorsal depressions at the sides and stand in a series along the back."

"Ventral series [Plate 7, Fig. 92] of protuberances marked off from infralateral series by a hiatus, by lower and thinner teeth, and by the forward arching of the series. It consists essentially of two stronger lateral teeth with a varying number of less prominent ones between. On the tenth segment the series is shortened and the middle elements are almost lacking. Between these arched series of teeth and posterior ridges on the ventral surface of the segments (which are represented by about four low round protuberances) are depressed areas similar to those of the dorsal surface. These are bounded at sides by

first protuberances of infralateral series. Each segment from fourth to tenth has a pair of impressed dots on inner pair of elevations of the posterior longitudinally striate ridge. Segments six to nine have four of these impressions, one outside of each of the median pair."

"On the last or eleventh segment the anus is a semicircle with the convex side downward, overhung by four prominent tubercles in bilaterally symmetrical pairs. Mouth of the air tube a smooth oval surface just above the anal tubercles. It has a vertical slit and is surrounded by a projecting crenulate frill."

Pupa (Plate 11, Fig. 121, *a*, *b*, *c*).—One of the full grown larvæ collected by Mr. Pergande pupated and a female imago issued May 29, 1899. McAtee's description follows.

"Length of the pupal shell 19 mm.; greatest diameter 6.5 mm. Head and thorax of the pupa a lighter, abdomen a darker ferruginous. Head and thorax finely and irregularly wrinkled; anterior half of each abdominal segment finely wrinkled transversely, posterior half with wrinkles less distinct or absent (especially on ventral surface), but very finely and closely punctuate. This makes the color appear more intense, in places almost orange. Middle of the segments, except the first, surrounded by an interrupted fringe of definitely grouped sharp pointed spines, the larger of which tend to be serrate."

"Vertex of head marked by a narrow rounded longitudinally wrinkled transverse ridge. In the depression between this ridge and the antennal prominences and in front of the extremities of the ridge are two outwardly directed setæ. Antennal sheaths short, appressed, downwardly curved, conical, arising from two low wrinkly protuberances (the antennal prominences above mentioned). These prominences separated by a deep fold, and from them curved and diverging impressed lines run down the face. Below each antennal sheath is a widely separated vertical pair of setæ."

"Prothorax longitudinally wrinkled, except for a smooth area behind and below each antennal sheath. A setiferous tubercle stands above each of these smooth areas. Prothorax angulate in the median line behind. Mesothorax three times as long as the prothorax, bearing two spiracular tubercles near anterior lateral angles (about opposite the middle of each lateral half of the prothorax). These tubercles similar to those described below, but complicated by flexures of the walls. They bear at the summit upwardly arched crescent-shaped rimæ or air slits. The only setæ I can find on the mesothorax are one on each side directly back of these spiracular tubercles. Metathorax very short in the median line, but somewhat longer at the sides, which have two rounded angles anteriorly. Wing pads and leg sheaths, the latter slightly the longer, almost covering ventral surface of the first abdominal segment."

"Middle of each side of first to seventh abdominal segments with elevated round polished knobs bearing on the posterior portion of their summits the pos-

teriorly arched crescentic spiracles. Second to seventh segments with a sharp pointed backwardly curved spine directly posterior to each spiracular tubercle. A short distance above this spine is a similar one, and between these two from one to four shorter ones. Some shorter teeth occur also both ventrally and dorsally from the stout spines. These lateral spines become stronger posteriorly."

"There is a definite break between the groups of lateral spines and the weak spines forming the lateral elements of the dorsal series. This break is marked by sharply impressed lines on segments 2 to 7 [Plate 11, Fig. 121]. Dorsal series of spines on second to seventh segments consisting of a pair of stout spines on each side of the median line [Plate 11, Fig. 121], the pair on the seventh segment being most widely separated. On each side of the mid-dorsal pair are about three other symmetrically placed strong spines. Between the larger spines are varying numbers of shorter ones, and gradually diminishing small ones terminate the series on each side. All spines sharp pointed and curved backward. Ventral surface of the second to seventh segments with smaller spines, having median pairs of stronger teeth, most widely separated on the third segment and nearest together on the seventh. There is a tendency for one of the minor spines on each side of the median pair to be larger than its fellows. These smaller spines of varying number but maintaining their series across venter of the segments, interrupted only by the stronger ones and diminishing gradually on each side. A wide hiatus exists between the last of the ventral series on each segment and the group of spines near the spiracular tubercle. Eighth or terminal segments with three strong spines on each side, connected by series of weaker points [Plate 11, Fig. 121]. The pair made by the uppermost of these strong lateral teeth is more widely separated than the corresponding ventral pair. In each case the interspace (that is, the dorsal and the ventral area of the segment) is devoid of points, except for small ones immediately adjacent to the large spines. The location of the larval anus is marked by a rounded transversely wrinkled knob and the spiracular eminence consists of two conspicuous tuberculate projections surmounted by sharp pointed downwardly curved spurs."

This is, so far, the longest description of a tabanid larva and pupa which we possess.

Of early stages of the many exotic Pangoniinæ nothing appears to be known.

HÆMATOPOTA, EARLY STAGES IN GENERAL.

We owe to Neave notes on the early stages of this genus. He collected at Mt. Mlanje in southern Nyasaland, Africa. The larvæ were by no means easy to obtain as compared with those of *Chrysops*, a few individuals of three species only being found in September and October. Later in the season, in January, considerable numbers of the larvæ of an unidentified species were obtained, perhaps those of *Hæmatopota insatiabilis* or an allied species. The larvæ all seem to resemble each other closely and it is very difficult to distinguish specific differences in them, though they differ in a marked manner from those of any other genus of tabanids seen by Neave. The differences between these larvæ and those of *Chrysops* are discussed under that genus (Plate 5, Figs. 73 and 74). The limitation of the pigmented areas to the anal segment and the abruptly truncated syphon seem to be characteristic of and peculiar to this genus, as also are the very short, sometimes almost invisible pseudopodia.

The genus *Hæmatopota* is represented by a large number of species in the Old World, especially in tropical Africa; the species are less numerous in the New World and the genus is entirely absent in Australia.

Hæmatopota crudelis Austen.—An African species, originally described from German East Africa, and by Neave found by no means uncommon near Mt. Mlanje, in southern Nyasaland, in the months of October and November.

The first larvæ of *Hæmatopota* found in Africa, by Neave, belonged to this species. Several of the larvæ were found in September and October, and, though many were lost, two males and three females eventually emerged in October and November.

The larva, which is figured (Plate 6, Fig. 85) differs from that of *Hæmatopota insatiabilis* only in that the pigmented areas are more pronounced round the anus and at the base of the syphon.

The pupal aster (Plate 13, Fig. 170) has hooks of nearly equal length and forms a very regular star. There is no true dorsolateral comb, though one female has a minute knob-like process in place of it. The pupal aster is figured.

Hæmatopota decora Walker.—An African species, which prefers low and dry country, therefore not common in the Mlanje district of southern Nyasaland, where Neave made his studies. However, Neave has acquainted us with the larval and pupal stages.

The larva was of the usual *Hæmatopota* type, but the pigmented areas on the anal segment were an orange color. This might, however, have been due to the mature condition of the larva, which was about to pupate. The pupal aster is regular in shape. A dorsolateral comb consisting of three spines is present. Pupal aster and dorsolateral comb of a female are figured (Plate 13, Fig. 169, *a*, *b*).

Hæmatopota insatiabilis Austen.—An African species, abundant near Mt. Mlanje, southern Nyasaland, about November and early December, according to Neave (1915).

A few larvæ of *Hæmatopota insatiabilis* were obtained, and from some of these, three females were bred between November 7 and 10, 1913. The three posterior segments of "what is believed to be the larva of this species" are figured (Plate 6, Fig. 86). There seems to be no pigmentation except a narrow double ring at the posterior end of the eleventh segment. The circular ridge on the tenth segment shows no pigmentation. The twelfth segment is very short and rounded.

The pupa is figured (Plate 11, Fig. 131) but not described. The pupal aster of this species (Plate 13, Fig. 172, *a*, *b*, *c*) is very remarkable, the upper hooks being reduced to mere knobs, while the middle pair are enormously enlarged. A well marked dorsolateral comb is present. Pupal aster of the female in side view and from behind is figured, also the dorsolateral comb.

Hæmatopota pluvialis Linné.—One of the commonest and most widely spread tabanids in Europe, known also to torment the reindeer in Lapland, where it occurs in incredible numbers. It is the representative of a genus spread all over the world but absent in Australia, while in Africa it is represented by a large number of species.

In the more recent literature nothing is found concerning the life history of this species, the earlier work having escaped attention of recent investigators.

Zetterstedt believed that the larvæ of *Hæmatopota* live in the dung (Scholtz).

Scholtz reported, as early as 1850, having found the pupæ of this species; we shall give his statement with the other facts available on the pupal stage.

Larva and pupa of this species have subsequently been described by three authors, independent of each other. All agree that they are terrestrial in habit.

Brauer (1883) once obtained the larva of *Hæmatopota* from the larva of *Helops lanipes* (Coleoptera, Tenebrionidæ), from which when it seemed to be in process of molting, the tabanid larva came to light.

Brauer (1869) is the first to give accurate descriptions of the early stages (Plate 6, Fig. 83, *a–d*), with the exception, however, of the egg state, which remains unknown in *Hæmatopota pluvialis*, while it has been observed in other species of *Hæmatopota*.

Brauer found, in an excursion made in June, 1869, to Lang Enzersdorf, Austria, near the railroad embankment, in a region of scattered cottonwood (*Populus alba*), in entirely dry soil, a white grub, about 20 mm. in length and 3 to 4 mm. in diameter, which was unfortunately only briefly examined with a lens. When in the evening Brauer began to examine it more closely the grub had pupated, and it was only from the remainder of the larval skin that the character of the larva could be ascertained. However, the description obtained from this meager material is very accurate.

The larva (Plate 6, Figs. 83, *a*, *b*, 84, and 91) is cylindric, twelve-segmented (head included). The head (Plate 6, Figs. 84 and 91) is formed after the type of tabanid larvæ, almost completely differentiated, the chitinous plates diverging behind and exceeded in length by two long rods in the middle, which are continuous with the middle chitinous plate of the head and with the labrum. The latter is narrow, somewhat curved downwards, widened at the tip, and ciliated laterally. Close to the sides are the hooked, downwardly curved parallel mandibles, their margin being convex and serrate. Below the mandibles and partly within the concavity formed by them are the maxillæ, which are soft in appearance. Their basal part is globular and slightly spiny, the distal part is formed by a finger-like piece; at the side of the latter, externally, a two-jointed palpus of twice its length, the two joints being of equal length. The terminal joint, moreover, is widened and excavated to spoon shape. Above and at the sides of the mandibles are the antennæ, of which the distal joints are cylindric and simple,

the first one thick and long, the second short and narrow. There are no bristles at the base of the antennæ. The eye-spots are small, perhaps more distinct in life, and situated behind the middle of the head. The whole head is deeply retractile. The body is pure white in color with many longitudinal striæ, and bearing, on the fourth to tenth segments, small fleshy warts on the lateral and ventral sides, each segment having four. These warts can be retracted, giving the larva an almost smooth appearance. The last segment ventrally with a thick half globular anal prominence, and posteriorly with a short conical structure with a vertical two-lipped fissure. The lips of the latter are strongly chitinized, transversely sulcated, each of them leading to one of the main tracheal trunks; they represent the posterior spiracles of the larva, while the small anterior spiracles are situated on the posterior half of the second segment.

The whole structure of these respiratory organs indicates that the larva had not lived in water or left it only for pupation, as, according to Brauer, in all tabanid larvæ of terrestrial habitat the posterior spiracles are analogous in structure, while the aquatic larvæ of *Tabanus autumnalis* and *Hexatoma pellucens* are enabled to extend the last segment like a telescope, showing a similar fissure only at the end of this tube.

A much more detailed description of this larva is given by Perris (1870), who found it twice, and each time in a single individual, in the decayed wood of old pine stumps. Of the two specimens found one was sacrificed to be studied; the second one metamorphosed and produced, to the surprise of the observer, the species *Hæmatopota pluvialis*. This fly, as he says, is very common in regions of France where there are no pines, and so abundant in the Landes that he could not have missed the larvæ if the stumps or the bark of dead trees were its normal place of development, as he had especially studied all insects inhabiting the pine. Consequently, Perris assumes that the insect has deviated from its ordinary habits. However, the larvæ were found in the pine wood detritus itself where they apparently had lived, and in which the larva mentioned changed to pupa after three months.

A translation of Perris' description of the larva (Plate 6, Fig. 82, *a–e*) is given below:

Larva (figured) 12 mm. in length. Smooth, hard, leathery (coriaceous), cylindric, attenuated at both ends, anteriorly more than posteriorly, very finely and densely striated in a longitudinal direction.

Under ordinary conditions the head, which is small in comparison with the body, reddish and semicoriaceous, appears conical; but when protruded forward, it forms a sort of neck posteriorly. It is retractile to the extent that it can disappear completely in the first segment. The front is protruded into a fleshy mouthpiece which Perris assumes to be the labrum. To the right and to the left of this a black horny linear piece is seen, which is curved anteriorly and prolonged posteriorly into a rod, becoming more and more thinned out, which is observed through the transparent tissues and which is inserted deep in the first segment. These two rods are at first parallel, then somewhat diverging. They serve, according to Perris, as places of attachment to the muscles which move the head and the linear piece mentioned, which are the mandibles. Below, two fleshy subconical pieces are seen, fused together at their base, of which the inner is separated from the outer one only at the tip of the latter. The external piece, which is a maxilla, is surmounted by a rather long palpus of two joints, the first of these joints one and a half times as long as the second; the inner piece is the lobe of the maxilla. Above, on each side of the head, opposite to the base of the maxillæ, a rather long antenna is situated, which appears to consist of three joints, of which the first is very short, the second four or five times longer, the third slender and about one-half as long as the preceding.

This larva has, consequently, a protruded labrum, two horny serrate mandibles, two maxillæ with their lobes, two maxillary palpi consisting of two joints, two antennæ consisting of three joints, all extending beyond the head.

The body is formed of eleven segments; the first, or prothorax, truncated conical, is one and a half times as long as all the other thoracic segments, which are slightly shorter than the abdominal segments. The first six of the latter have eight fleshy and retractile protuberances, two dorsal ones distant from one another, two on each side placed close together, and two ventral ones placed at a distance from one another like the dorsal ones. These protuberances are of great help to the larva when it starts to walk. In this act, the larva presses the head against the surface on which it is to move and contracts the body, at the same time retracting the protuberances; then, freeing the head and exserting the protuberances which, all around, exercise a strong pressure, it pushes forward. The last segment of the abdomen, which is somewhat rounded at the sides, is rather suddenly and considerably narrowed down posteriorly, where it terminates in a disk in the center of which a reddish vertical slit is observed with the aid of a lens. At high magnification this slit is recognized to be a spiracle the lips of which are transversely striated. In seeking for the remaining respiratory orifices, two of these oval and reddish spiracles are found at the posterior border of the prothoracic segment, one on each side. Under the last segment, in its anterior third, there exists a fleshy prominence, of the shape of an ellipsoid placed transversely, and retractile; it is traversed by a longitudinal fissure or sulcus which indicates the anus. This prominence also serves to aid locomotion.

The body of the larva is completely covered, except in the anal region, with very fine and densely placed longitudinal striæ easily seen with the lens. They certainly play a part in the contractions and dilatations of the body.

The larvæ of this species have been found and described again by Beling (1875). One larva was obtained by him on March 24, 1873, from hog dung which had been collected from a meadow and piled up at the edge. This larva was kept in a room which was not heated, and pupated on June 24, producing a male imago on July 6, after a resting period of twelve days. From December, 1873, Beling frequently found single larvæ in the soil of cultivated fields, several times also in the earth of hedges bordering on meadows. The larvæ which were collected were kept in a glass jar partly filled with humus and continued to live for months in this medium, without injuring one another. However, at the time when metamorphosis was to take place, they killed one another, apparently for the purpose of feeding on the killed individuals by sucking out their contents, and of the ten larvæ finally only one remained, which pupated on June 25, 1874, and produced a female imago on July 5, after 11 days of pupal condition.

According to Beling, the larvæ resemble closely those of *Tabanus bromius* in color, appearance, and habits, but are at once distinguished by being shorter and thinner, and by the much finer punctated[12] longitudinal striation. Concerning localities of capture, Beling states that the *Hæmatopota* larvæ occur chiefly in humus of cultivated fields, while the larvæ of the terrestrial *Tabanus* species mentioned are found usually in grass-covered or meadow soil. Beling's description of the larva is translated below.

Larva.—10 to 12 mm. in length, 2.5 mm. in width, somewhat contractile; when fully extended, up to 15 mm. and even more in length, twelve-segmented, pale yellow, strongly and glassy shining, with very fine irregular (punctated) longitudinal striation, somewhat narrowed in front and behind, and especially during locomotion narrowed down to spindle shape in the anterior part, with small narrow brown head completely retractile into the first segments, appearing, when seen from the dorsal side of the first two segments, as a brown longitudinal stripe, broadened behind and ending in two points. The last body segment is shorter and narrower than the preceding one, rounded at the posterior border,

[12] The German word used is "*Nadelrissig.*"

with a cushion-like prominence on the middle of the rounded part. The prominence is often in the shape of a short thick cylinder, truncated at right angles, bearing in the middle the vertically placed stigmatal fissure. Anus prominent on the under side of the last segment. On the under side of the fifth to the eleventh segments inclusive are transverse locomotor swellings near the anterior margin, forming six longitudinal rows, of which four on the ventral side are arranged in two pairs, not far distant from one another, while in the outer rows ridges are placed at a greater distance from one another and are also placed farther back from the anterior segmental border.

Scholtz (1850) reports having found the pupæ of this species, together with those of *Tabanus autumnalis* and *Tabanus tropicus*, on an excursion with Prof. von Siebold and Dr. von Frantzius in the neighborhood of Breslau, in 1850, at the edge of a pond which was entirely covered with *Lemna*, and the water of which was polluted from manure piles surrounding it. Here the pupæ were found quite near the edge under a thick moist mass of decaying *Lemna*, together with the pupæ of *Stratiomys*, *Syrphus*, etc.

A translation of Brauer's description (1869) is as follows:

The pupa (Plate 6, Fig. 83, *c*, *d*) 15 mm. in length, slender, with no spines at the anterior end, bearing here only two small tubercles. The wing and leg cases do not extend beyond the first abdominal segment; the second to tenth abdominal segments bear a circular armament of bristles, the last segment ending with a thick and only slightly diverging fork which is more or less embedded in the cast larval skin.

From Brauer's pupa there appeared, after two weeks, a male of the species in question.

More precise in detail is the description given by Perris (1870), obtained from the cast pupal skin:

The pupa (Plate 6, Fig. 82, *f*, *g*) presents all the parts of the perfect insect. The organs of the head can be guessed rather than seen. Two frontal tubercles (trouqués), surmounted by a bristle, seem to be the cases for the antennal bases; two other oblique tubercles overlapping with their borders, in the region of the mouth, seem to hide the palpi. Between these two tubercles and those preceding them two patterns are formed by small elevated lines, the upper ones forming a parenthesis, and the lower ones a sort of brace. Exterior to these patterns there exists, on both sides, a transversely triangular prominence, with its summit overlapping a little the border of the head. I (Perris) consider these prominences, to judge from their location and form, as the cases for the distant

division of the antennæ. Below there are two small tubercles placed obliquely one below the other and surmounted by a little hair, and on the vertex four tubercles arranged transversely, the outer ones larger and bearing a hair. The dorsal part and the sides of the thorax bear some small hairs. The abdomen, consisting of seven segments, presents on the posterior thirds of each of the first six a circular series of stiff bristles, unequal in length, thick at their base and directed backwards. The last segment is small and ends in a small crown of six conical diverging teeth, the two upper ones a little smaller than the others. Rather near the anterior border of the thorax two obtuse protuberances are seen, somewhat more excavated and striated at the summit, crossed by an oblique furrow and inside showing a depression, the thoracic spiracles of the nymph. There are also visible the very conspicuous marks of six abdominal spiracles; they are situated laterally on the first six segments.

It is conceivable that the tubercles, the hairs, and the bristles with which this pupa is armed, are very useful when it wants to move or to reach the open air in order that the perfect insect should not be hindered in the act of eclosion. In the eclosion the shell of the pupa splits all along the median and dorsal side of the thorax.

Beling also describes the pupa of *Hæmatopota pluvialis*.

Pupa.—10 to 13 mm. in length, about 2.2 mm. in width, cylindrically rounded, of a dirty brownish yellow color, slightly shining, somewhat narrowed behind. Below the two small front teeth placed beside one another, and above the frontal margin two small tubercles, not much farther distant from one another, and each bearing a long bristle. Above this tubercle, somewhat distant from it, is another separate hair, and, in addition, on each side of the thorax are two hairs, one directly located behind the other. Abdomen nine-segmented, first segment very short, hardly reaching to the fourth part of the length of the second segment; third to eighth segments inclusively surrounded, near the posterior margin, with a circle of rather densely placed brownish bristle-shaped teeth, of unequal length, appressed backwards, and shorter on the ventral than on the dorsal side. Last abdominal segment small, bearing in the middle of its under surface a row of similar bristle-like teeth as the other segments, and at the end with six outwardly divergent comparatively strong teeth with blackish tips, arranged in a hexagon of which the upper two are shorter and weaker than the remaining ones.

Hæmatopota sp.—Two undetermined species of *Hæmatopota* were observed by Patton and Cragg (1913) in Madras, to oviposit, as all the small tabanids observed by these authors, "on blades of grass just at the edge of a shallow stream, or on the leaves of the lotus plant at the edges of small ponds, but never over deep water."

These species of *Hæmatopota* would then fall under the heading of those larvæ which, according to Patton and Cragg, have no air sacs and, consequently, perish when falling into deep water. Like the other small species, these *Hæmatopota* "spread out their eggs in one or more layers on blades of grass." The eggs of one species of *Hæmatopota* are dark gray when deposited, according to the same authors. No descriptions or figures are given. The eggs are said to be torpedo-shaped (not cylindric) in all the smaller tabanids in Madras, which should hold good for *Hæmatopota*.

Hexatoma pellucens Linné.—A European species, according to Schiner common in Austria, attacking man as well as animals.

In the middle of June, 1868, in a garden in Breitensee, Austria, Ernst Marno found in a reservoir 8 feet deep, filled with rain water and dung fluid, together with larvæ of *Eristalis* and *Culex*, some dipterous larvæ of about 25 mm. in length, which were on the dorsal side beautifully mottled with black. Some of them kept in this water had died the following morning and were preserved in alcohol. Brauer, who was consulted, pronounced them to be tabanid larvæ of an unknown species.

Early in July, after a heavy rain, larvæ were again found, which in color and appearance seemed to be the same species, and of which in the middle of July when they apparently were full grown, a considerable number were collected from the mud of their habitation.

Of these, however, many perished which were continually replaced by new captures. On this occasion also empty pupal cases and some full ones were found; the latter had already made some progress in the transformation to the perfect insect, but had fallen into the water and had soon died and decayed.

Brauer determined the contents of these pupæ, as well as a dead fly which was found in the water, as belonging to *Hexatoma*. However, a search for the pupæ in the soil surrounding the reservoir was in vain, and there remained some doubt as to the identity of the larvæ and pupæ.

The larvæ captured by Marno were kept by him in a shallow flower pot, which was filled half with moist earth and half with moist decaying foliage, as it had been observed that the larvæ ap-

parently showed a preference for this environment and had been seen to work their way into a decaying leaf. They did well under these conditions; only a few of them died, and on July 22, in the afternoon, the first soft pupa was found, the larval skin still adhering to it, and identical with those found dead in the water.

A few days later, when a search was again made for the larvæ, none of them were found, but instead it was discovered that they pupated in the interspaces of the somewhat defective brick walls of the reservoir, and here some pupæ as well as larvæ were collected, of which the latter were more or less contracted in preparation for their metamorphosis. From these pupæ the first fly was obtained on July 27, in the morning.

The duration of the larval stage is about three weeks; the duration of the pupal stage Marno could not ascertain, leaving his material to Brauer to study.

The larva (Plate 6, Figs. 89, *a*, *b*, and 90, *a*, *b*, *c*) has been described by Brauer (1883), who reports it to be aquatic, being found in pools and puddles. A translation of Brauer's description is given below.

Larva, when extended, slender and spindle-shaped; when contracted, obtusely cylindric, the anterior and posterior segments being retracted in the latter case. The body consists of a head capsule and eleven segments. The cuticle has a striped appearance because of the many closely arranged longitudinal furrows. The first three segments and the under surface are bone-white (yellowish white), the dorsal surface of the remaining segments shows beautiful grayish brown patterns on a white background, brought about by microscopic pubescence. The head capsule (Plate 6, Fig. 90, *a*, *b*, *c*) is narrow, formed like that of all tabanid larvæ, compressed, and can be retracted into the second segment. It consists of four plates which posteriorly are not connected, and of which the two middle ones are drawn out into long rods. The lateral plates show on half their length on the outside an irregularly rounded and convex eye-spot on each side. At the anterior end is a comb-like upper lip reaching above and between the mandibles. On each side of its base there is a cushion-like projection thickly beset with mostly two-pointed bristles, on which the antennæ are inserted, which are short and two-jointed. The first joint is cylindric, the second divided lengthwise into two parts. The exterior point is shorter than the interior. Below and to the inside of the antennæ are the hooked and more strongly chitinized mandibles, which may be moved up and down parallel to one another. Their surface is transversely sulcate; consequently the anterior convex margin is serrately incised.

Within the concavity of the mandibles the softer maxillæ are placed. The extremity of the latter, curved hook-like in the same way, runs parallel with the mandibles, so that to the observer from the side, two hooks appear superposed one on the other. The maxillary palpus projects obliquely towards the outside and above, and has a thick cylindric basal joint and a thinner simple terminal joint of double length. Beginning with the third body segment, there appears a short intermediate segment, on which rather long bristly hairs are inserted. On the ventral side several circular protuberances with bristles are noticed on the intermediate segment.[13] The fourth to tenth segments show on the dorsal side two, and on each side one spinous protuberance. The last segment has, on the ventral side, the paired anal prominence, covered with finer and stronger hairs and surrounded by a three-sided furrow.

At the basal angles of the anal prominence small wart-like swellings appear. From the last segment a respiratory tube may be extended which is rather sharply pointed and narrowly cone-shaped, showing at the tip a vertical fissure between two thickenings, the endings of the main tracheæ of the metapneustic larva.

The pupa is a free pupa, and is found in the ground.

The head of the pupa bears on each side above the base of the antenna a small wart with a bristle, and above and below the antennal sheath, on the eyes, similar but slightly smaller warts. Behind the prothorax are the anterior spiracles, placed on small cylindric prominences which do not project far. The wing and leg cases reach the posterior margin of the first abdominal segment.

Length of the larva: 27 to 30 mm.; width in the middle 4 mm.

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[13] The German word used here is "*Zwischenwulst*" which means a swelling lying between the segments.

TABANUS, EARLY STAGES IN GENERAL.

Much that can be said about *Tabanus* in general has been said already under the family Tabanidæ.

The eggs of *Tabanus* are not appreciably different from those of *Chrysops*, except that the latter resemble, according to Patton and Cragg, more those of the smaller species of *Tabanus*, the eggs of the larger species being of slightly different form. According to Hart, the eggs of *Tabanus* are always laid in clusters composed of several layers, but recent observations have shown that from this rule exceptions are frequently met with, when the eggs are spread out in one layer or even loosely scattered. As a rule, however, the eggs are laid in a cone-shaped or bilaterally symmetrical mass containing several layers of eggs and attached to various objects as specified under the heading of Tabanidæ.

The larvæ and pupæ, studied by Hart, form two groups, which may be called the *lineola* and the *atratus* groups. The larvæ show the most distinctive characteristics in the structure of the surface especially of the prothorax, which has lateral, dorsal, and ventral shining areas, limited in front by an opaque pubescent annulus. Comparing the anterior extension of these shining areas, Hart found them all of about the same length in *Chrysops* and the *lineola* group of *Tabanus*, although this length varies in *Chrysops*. The lateral prothoracic areas are extensively invaded by the anterior annulus in the *atratus* group of larvæ, the striated shining space becoming basal, being not more than half as long as the dorsal area. The upper lateral space of the mesothorax is not closely striate, and is quite shining. In all except some of the *atratus* group the remaining lateral striation, including that of the prothorax, is not much finer, and is also shining, but in the others, as in *atratus*, the prothoracic and abdominal lateral striation becomes microscopically fine and even subopaque. A smooth spot near the lower hind angle of the prothoracic lateral area furnishes another good differential character. The dorsal and ventral striation varies in extent according to age, but the thorax is striated above in *Chrysops*, and smooth or nearly

so in *Tabanus*. The three types of coloration observed are well shown by the three *Tabanus* larvæ figured (Plate 3, Figs. 40, 41, and 42).

Pupæ.—The great difference between the abdominal spiracles and terminal teeth of the *lineola* group and those of the *atratus* group is evident from Hart's figures (Plate 13, Figs. 162 and 163). In both, the thoracic spiracles are in a plane nearly parallel to the adjacent surface and the spinous fringes contain long and short spines. The preanal fringe in the *lineola* group shows more or less of a chitinous web, uniting the bases of the spines.

A pair of short appressed palpal sheaths on the lower surface of the head, resembling the antennal sheaths, differs slightly in position in related species of the *atratus* group.

The species of *Tabanus* have been classified by various authors, but for convenience, I have listed them here in alphabetical order.

We possess data on early stages of fifty-five determined, and fourteen undetermined species of *Tabanus*. Those of some of the worst stock pests still await description.

Tabanus albimedius Walker.—This is one of the commonest Indian species of *Tabanus*, according to Patton and Cragg, and occurs almost all the year round in Madras.

Observations on the life history and early stages have been made by Patton and Cragg (1913). The species oviposits in a variety of situations, but most frequently on the leaves of some plant overhanging deep water. The egg masses have also been found on small rocks in the bed of a stream, and on pieces of string hanging over house drains; on one occasion a mass was found on a papaya tree, at the foot of which water was occasionally allowed to flow. The number of eggs laid is between 500 and 600. The eggs, as in all the larger Indian species mentioned by Patton and Cragg (*Tabanus striatus*, *albimedius*, and *speciosus*) are subcylindric with tapering ends. The eggs of *Tabanus albimedius* measure 1.9 to 2 mm. in length and 4 mm. in breadth. The eggs are brownish white when deposited A chalcid parasite, which was not identified by Patton and Cragg, regularly destroys large numbers of egg masses of this species, and of *striatus*. This species, as the larger species of India in

general, oviposits any time during the day, but as a rule in the morning.

When "one of the larger species" (including *Tabanus albimedius*) is about to oviposit "it alights on the leaf or blade of grass with its head downwards; it then thrusts the tip of the abdomen forwards under its thorax, and deposits an egg, which adheres to the leaf owing to the sticky substance which accompanies it. The abdomen is then returned to its original position, and as soon as the next egg is ready to be laid, is again flexed, and the second egg is placed at one or other side of the first. In this way three or four eggs are laid on one side of the first and three or four on the other. The mass at this stage has the shape of a V. The fly now moves forwards, and, raising the end of the abdomen to one arm of the V, places a number of eggs down the side until the apex is reached; she then changes over to the other side and deposits eggs all down that arm up to the apex. In the end a raised compact mass of eggs is built up, which, if examined with a lens, demonstrates the precision with which the eggs are placed in reference to one another."

The larva of *Tabanus albimedius* has not been described and also not figured, by Patton and Cragg, though they evidently had it under observation. A good characterization of tabanid larvæ in general is given, but the differences of the various species are not entered upon.

Of the larva of this species, by the same authors, dissections have also been made, and detailed illustrations are given of the mouth-parts (Plate 7, Fig. 97), and of the alimentary tract (Plate 7, Fig. 98).

The figure given of the head shows the mouth appendages: labrum, maxillary palpus, maxilla, antenna, and mandible; also the lateral area of spines behind the antennæ. On the figure the antennæ appear to be two-jointed, while the authors state that they are three-jointed in all tabanids.

The figure given of the alimentary tract shows the esophagus, proventriculus, the narrow salivary glands, apparently inserted in the pharynx, the mid gut, hind gut, and Malpighian vessels, four in number.

Patton and Cragg were the first to publish anything on the alimentary tract of a tabanid larva based on dissection.

Tabanus atratus Fabricius.—A species inhabiting eastern North America, as far west as Colorado, conspicuous by its large size and

uniformly black color. About its early stages we are informed by Walsh, C. V. Riley, Hart, and Hine. As Hine summarizes, the eggs are deposited around marshy places on grass and sedges, and the larvæ are found by digging in the mud.

Hart (1895) is the first to give notes about its oviposition, which was noticed in Illinois, in July and August, and egg masses then became frequent. The last date for the imago was August 15. On August 11, a female was observed ovipositing on the side of a wooden frame standing over the water. The egg mass was placed in a breeding cage, and one week later, on the 18th, many larvæ hatched from it. Another egg mass of the same form and appearance, placed on the dark bark of a stick projecting from the water, was brought in July 27, from which hatched on August 4 larvæ apparently of this species.

Hart's description of the egg mass and egg is given below:

"*Egg Mass.*—Blackish-brown, subconic, with oval base, 10–15 mm. long and 8–10 mm. wide, height 5–7 mm.; sides convex or concave, apex correspondingly rounded or pointed; eggs pointing obliquely upward and towards one end, both sides meeting upon that end in a more or less prominent longitudinal crest. The eggs are stacked in four or five tiers, one above another, and gummed together into a firm mass."

"*Egg.*—Length 2.5–2.7 mm., diameter 0.4 mm. Dark brown, subcylindrical, ends more or less tapering and curved, surface minutely rugose and subopaque."

Hine completed these observations in 1906. According to him, the eggs of *Tabanus atratus* are generally placed in masses of various sizes on the leaves and stems of grasses and sedges and other plants growing in marshy or wet ground, but not necessarily in the water. A single mass may contain as many as 500 eggs, but often they are smaller and they may be larger; they are white when first placed, but soon turn brownish. The mass is very convex and composed of several layers, one above the other, the bottom layer being attached to the surface of the leaf or stem and the other layers each to the one that was placed before it. Each egg is elongate, spindle-shaped, between 2 and 3 mm. in length, and narrowed at each end. A female was observed, in Ohio, ovipositing on June 23 at 11 o'clock. The eggs were taken and kept in a room out of the sun, where they hatched on the morning of July 2 before 6 o'clock, thus requiring an incubation

period of nearly nine full days. It has been proven that the eggs of tabanids hatch more quickly when exposed to the sun during the day (see *Chrysops*), where they are usually deposited; therefore the time given is probably too long for eggs under natural conditions.

There is no definite way, as far as our knowledge goes, according to Hine, of telling the eggs of the black horse-fly from those of other species of this genus, but being a large species the masses are much larger than in some others, and are more convex than usual. The particular place of oviposition is in a measure characteristic.

It is of interest to note that the egg masses of *Tabanus atratus* are often found to be parasitized.

When the two egg masses mentioned and described by Hart had produced larvæ they were placed in a dry vial, and a little later it became evident that both masses had been parasitized by Hymenoptera, minute black imagos emerging freely in the vial. An examination of one of the masses showed that about one-half of the eggs had been infested. Examples of the imago were sent to Mr. W. H. Ashmead, who found the species to be a new one. It was described by him as *Phanurus tabanivorus* (Ashmead).

I give the description of this insect at the end of the chapter on *Tabanus*, under the heading, Parisites of the early stages of Tabanidæ. The illustration of this insect, given by Hart, has been omitted.

Hart is also the first to tell us about the early larval stages of *Tabanus atratus*. From the two egg masses he obtained larvæ hatched as already stated, on August 4 and on August 18. In the latter case, the incubation period was determined to be one week. The larvæ were at this time commonly found in water among the vegetation, less commonly in the sand of the shore, and young individuals became frequent.

Larva, Newly Hatched (According to Hart).—"In this stage the lateral areas are sculptured similarly to those of the adult, but the dorsal and ventral areas, though shining, are rather sparsely striated. Traces of the dark markings are visible, especially on the posterior segments."

According to Hine, the larvæ, when first hatched, are about 3 mm. in length, white, and with a narrow darker shade at the union of each two segments. As soon as they drop to the ground they begin

to burrow and are soon beneath the surface, where they cannot be seen. At first these larvæ are very hard to see on account of their small size; consequently not much has been learned of their habits under natural conditions; but when they are nearly grown they are to be found in a variety of places. We now come to a discussion of the adult larval stage.

Walsh (1863) gives a description of an aquatic *Tabanus* larva which he was unable to identify as the imago obtained from this larva was in too bad a condition to be identified, having remained many weeks without care, in the breeding jar. From his descriptions, however, and also from his remarks on the imago, it is evident that the larva belongs to *Tabanus atratus*. There is in Massachusetts no other *Tabanus* known of this size and with wholly brownish black wings, and the description of the larva agrees with that given by later authors for undoubted *Tabanus atratus* larvæ. However, some of Walsh's smaller specimens may belong to *Tabanus stygius* or other species.

Walsh found his aquatic larva, on many different occasions, "amongst floating rejectamenta." On one occasion he found six or seven specimens in the interior of a floating log, so soft and rotten that it could be cut like cheese. Once he discovered a single specimen under a flat submerged stone, in a little running brook. Finally, once he met with one alive, under a log, on a piece of dry land which had been submerged two or three weeks before, whence it appears that it can exist a long time out of the water. Walsh had, on several previous occasions, failed to breed this larva to maturity, and the only imago he had was obtained in 1861 from larvæ which, suspecting them to be carnivorous from the very varied stations in which they had occurred, he had supplied with a number of fresh water mollusks, but the habits of which, in consequence of having been away from home, he was unable to watch. On September 2, 1863, he found a nearly full grown larva among floating rejectamenta, and between that date and September 23 this larva devoured "the mollusks of eleven univalves" (genus, *Planorbis*) from one-half to three-fourths of an inch in diameter; and on three separate occasions observed it work its way into the mouth of the shell. In this operation the pseudopods were energetically employed and Walsh found, on cracking the shells

after the larva had withdrawn, that a small portion of the tail end of the animal was left untouched—no doubt in consequence of his being unable to penetrate to the small end of the whorl in the shell—and also the skin of the remaining part and the horny tongue membrane.

Walsh's description of the larva of *Tabanus atratus*, including that of the imago which serves to establish its identity as to species, is given here:

"*Tabanus* —? Imago male. Blackish. Legs blackish, wings brownish fuscous. Length 0.70 inch. Expanse 1.30 inch. One decayed specimen came out between June 14 and July 14, 1861, from a larva found early in June."

"Larva, from the living specimens, obtained August 14, 1860, and September 2, 1863. Length 2.25 inches when extended, 1.75 inches when contracted; diameter 0.25 to 0.30 inch. The specimens found in 1863, 0.25 inch shorter. Body cylindrical, twelve-jointed, the three or four terminal joints much tapered at each side of the body, but more so anteriorly than posteriorly, and joints one and eleven, each with a retractile membranous prolongation at tip. Joints one to ten are subequal; eleven is about two-thirds as long as ten, and twelve about one-fourth as long, and 0.5 inch in diameter. Color a transparent greenish white, paler beneath, on the anterior and posterior margins of joints two to eleven, the anterior annulus laterally connected with the posterior by two to four dark green lines. On the dorsum of four to nine, and more obscurely on ten, a dark green basal triangle, extending half way to the tip; joint one with paler markings, and with no dark annulus behind; joint twelve entirely fuscous. Head small, apparently fleshy, pale, truncate-conical, 0.03 inch wide, and about 0.04 inch long in repose, inserted in joint one without any shoulder. The trophi occupy two-thirds of its length, but it has a long cylindrical internal prolongation, extending to the middle of joint two, which is sometimes partially exserted, so that the head becomes twice as long as before. All the trophi are pale and apparently fleshy except the mandibles, which are dark colored and evidently horny, and they have no perceptible motion in the living insect. The labium is slender, a little tapered, and three times as long as wide, on each side of and beneath which is a slender thorn-like decurved brown-black mandible. The labium resembles the labrum, but is shorter, and on each side of it is a slender palpiform but exarticulate maxilla, extending beyond the rest of the mouth in an oblique direction. No palpi. On the vertex are a pair of short fleshy exarticulate filiform antennæ, and there are no distinct eyes or ocelli. In the cast larval integument the entire head, 0.25 inch long, is exserted, and is dark colored and evidently horny, all the parts retaining their shape except the antennæ, labrum, and labium. The whole head has here the appearance of the basal part of the leaf of a grass plant, clasping the origin of the maxillæ on its posterior half, and

bifurcating into the somewhat tapered cylindrical mandibles on its anterior half. The maxillæ are traceable to two-thirds of the distance from the tip to the base of the head, scarcely tapering, bent obliquely downward at two-thirds of the way to their tip, and obliquely truncate at tip. On the anterior margin of ventral segments four to ten, in the living insect, is a row of six large fleshy roundish tubercular retractile pseudopods, the outside ones projecting laterally, and each at tip transversely striate and armed with stout bristly pubescence; on the anterior half of ventral joint eleven is a very large, transversely oval, fleshy, whitish, retractile proleg, with a deeply impressed, longitudinal stria. On the anterior margin of dorsal joints four to ten is a pair of smaller, transversely elongate, retractile, fleshy tubercles, covering nearly their entire width, armed like the pseudopods, but not so much elevated as they are. No appearance of any spiracles. Anus terminal, vertically slit, with a slender retractile thorn, 0.05 inch long, visible in 1860, but not in 1863. Head, and first segment or two, retractile."

When handled, the larva is, according to Walsh, "very vigorous and restless," and burrows with great strength between the fingers, and even on a smooth table walks as fast as any ordinary caterpillar, either backwards or forward; when placed in a vessel of water it swims vigorously, twice the length of its body at every stroke, by curving its tail around laterally, sometimes to the right, sometimes to the left, so as to touch the side of the fourth or fifth joint, and then suddenly lashing out with it. In such a vessel it always keeps close to the surface, and at the end of every stroke, and also when in repose, elevates the anal slit out of the water, on which occasion Walsh once saw a bubble of air attached to it. In the breeding jar the larva scarcely ever comes to the surface, but burrows among the decayed wood, aquatic plants, etc.

The larva described by Walsh differs, according to him, remarkably from the one described by Degeer (*Tabanus bovinus* L.), in having ventral pseudopods as well as dorsal ones. Walsh says that it might be supposed that the dorsal tubercles were branchial, "but for the fact that they are found in the earth inhabiting species described by Degeer," and that like the aquatic larvæ of *Prionoxyphon discoideus* Say (Coleoptera) it has a branchial apparatus issuing from the anus, and the short retractile anal thorn, observed in 1860, was the form assumed by this structure when out of the water. This assumption by Walsh is partly confirmed in so far as the terminal spine con-

tains the openings of the tracheal trunks. On the other hand, this spine has no connection with the anus, which is not terminal, as Walsh assumes, but lies in the fleshy tubercle of the eleventh segment, where Walsh observed "a deeply impressed, longitudinal stria."

The larva described by Walsh and which undoubtedly was *Tabanus atratus*, at least that specimen from which he obtained an imago, is found according to him from the beginning of June to the beginning of September, at which latter time he also met with a specimen only half the length of the full grown specimen (possibly belonging to a different species).

The pupa has also been described by Walsh, and the description is given here without change:

"The pupa (from the pupal integument) is cylindrical, suddenly rounded at the head, and tapering a little in the last two abdominal joints; the color is a very pale, semitransparent, yellowish brown. The mouth is represented by six tubercles, hexagonally arranged, above which, upon each side, is a trigonate three- or four-jointed antenna, pointing outwards. The pronotum commences immediately behind the antennæ and bears on its anterior dorsal submargin a pair of reniform tubercular spiracles, the mesonotum, to which the wing cases are attached, is twice as long as the pronotum, and bears on its anterior dorsal margin a pair of obliquely placed reniform tubercular spiracles, three times as long as the prothoracic ones. Then follows a very short metanotal piece, about one-seventh as long as the pronotum, bearing no spiracle, which is succeeded by eight subequal segments, all but the last bearing on their lateral dorsal surface a subbasal round tubercular spiracle. The first of these eight segments is simple and extends to the tip of the wing cases;[14] the others are all furnished two-thirds of the way to their tips with an annulus of appressed bristles directed backwards. The anal thorn is very robust, having a diameter of one-half the last abdominal segment, and is squarely truncate as soon as its length is half its width, and terminates in six small robust thorns, arranged in a regular hexagon. Length 0.97 inch; greatest diameter 0.21 inch. One specimen."

The second author to describe the larva of *Tabanus atratus* was C. V. Riley (1870). His description of the larva is quoted by Salmon and Stiles.[15]

[14] "I believe this first spiracle-bearing segment to be metanotal, as also the corresponding piece in the pupa of *Midas fulvipes*, u. v.," (Walsh).

[15] Salmon and Stiles, Emergency report on surra, pp. 100 and 101.

"The larva [Plate 3, Fig. 46] is a large twelve-jointed cylindrical affair, tapering at each end, of a transparent, highly polished, glassy, yellowish or greenish appearance, shaded with bluish green and furnished above and below as in the figure (figure given by Riley) with large roundish sponge-like tubercles which are retracted or exserted at the will of the insect. Though the external integument is so transparent that the internal structure is readily visible, yet this integument is firm and the larva most vigorous and active, burrowing with great strength either backward or forward in the earth and between one's fingers when it is being held. Placed in water it will swim vigorously by suddenly curling round and lashing out its tail, but it is apparently not as much at home in this element as in the moist earth, for it is restless and remains near the surface with the tip of its tail elevated into the air. When the water is foul it moves about actively near the surface, but when it is fresh it remains more quietly at the bottom."

This specimen, which Riley succeeded in breeding, was sent to him by Mr. Adolph Engelmann, of Shiloh, St. Clair County, Ill. It was found by Mr. William Cooper, of the same county, about ten feet from a small but permanent body of water. Mr. Cooper at first took it to be a leech, but when he attempted to catch it it immediately commenced burrowing into the ground.

The larva is declared by Riley to be semiaquatic, for it is at home either in moist earth or water. This specimen was kept for over two weeks in a large earthen jar of moist earth well supplied with earthworms. It manifested no desire to come to the surface. Riley found several pale dead worms in this jar, though not able to say positively whether they had been killed and sucked by this larva.

Hart (1895) reports that he has taken the larva of *Tabanus atratus* in every month of the season except June, at which time they had mostly reached the pupa or imago stage. They seemed to prefer the sandy shores, and were taken abundantly May 17 at Hart's Survey Station C, by running through a coarse sieve the surface layers of sand of the shore near the wave-washed margin. Station C is characterized by Hart as being located near the outlet of Quiver Lake, the shores being here near together and sheltered. The east bank was sandy, with a muddy coating over the part which is exposed at low water, while the west shore was of black mud grown over with willow trees and overflowed in moderately high water. The water was clear and on both sides thickly filled in summer with algæ and

other aquatic plants, having little or no current in ordinary stages. The same process of sifting was repeated June 25, and not a single larva was found. Individuals placed in breeding cages failed to transform. A pupa was collected June 30, from which the imago appeared July 17. A cast pupal skin was also picked up July 18. Several imagos were taken in the same locality between May 23 and June 22.

During the winter good sized larvæ sometimes occurred in dip-net collections, and March 18 they were again found to be common at Hart's Station C, in loose drifts, partly frozen, left by an early spring rise. The previous year they were common in April far from the margin, among sticks, logs, and other drift, marking the higher stage reached by the water on March 19 of that year. These situations remained moist for a long time, harboring a large variety of aquatic forms, some of which completed their transformations successfully while others apparently failed, the river remaining low and the weather dry.

Hart's description of the mature larva (Plate 3, Fig. 41) is as follows:

"*Larva, Mature.*—Length 45–55 mm., diameter 6–7 mm. Transparent whitish with a greenish tint, marked with conspicuous dark brownish or greenish fuscous, paler in younger specimens."

"Lateral prothoracic striated areas less than half as long as the dorsal, striation microscopically fine and opaque or scarcely shining, a small smooth spot on the anterior margin of the striated area, resting on the lower lateral line; remaining upper lateral areas of thorax much more coarsely and sparsely striate and shining; middle and lower thoracic areas—often much reduced, or even entirely covered by the lateral stripes—with distinctly finer and closer striation, but still shining; abdominal lateral areas with still finer striation, nearly as fine as that of the prothorax and feebly shining; dorsal and ventral areas all smooth and shining, rarely a few broken striæ about their margins, at the base of the prothorax or on the anal segment."

"Dark annuli distinct, broad, including false feet, transverse pale spot immediately in front of dorsal tubercles narrow or closed up in the mature larva; on the abdomen above, each annulus usually extends back on the median line in a triangular prolongation, often nearly attaining the next annulus, less developed in younger larvæ. Prothoracic lateral space occupied in front of the striated area by a dark opaque quadrate spot, extending from the anterior annulus. Lateral stripes of meso- and metathorax broad, at least the upper ones widened poste-

riorly, the lateral edges of the dorsal areas therefore parallel behind the middle of the segment, as seen from above; lateral stripes of abdomen, especially the intermediate ones, more or less abbreviated and broken up posteriorly except on the segment next the last. In these stripes the punctures of the upper and lower rows are indicated by rounded pale dots, and those of the inner rows by elongate dots. Last segment with broad dark annuli about base of respiratory tube and around anal prominence, with lateral connections; also more or less invaded above by the basal annulus, often leaving there only a pair of pale spots posteriorly. Often a dark spot in the anterior angles of the ventral space on the seventh abdominal, and one behind the anal dark ring."

"False feet moderately elevated, with coarse whitish pubescence more or less tipped with fuscous or with brownish in younger larvæ, dorsal pair narrowly connected over median line. Main internal tracheæ usually subparallel, sinuated, not very conspicuous, although easily traceable. Stigmatal spine rarely visible."

A few larvæ have been obtained by Hart, which are like *atratus* except in one particular—the surface of the body, especially of the anterior abdominal segments, shows a fine undulate wrinkling resembling the sculpture of the pupa, but smoother. As the specimens showing this appearance were shrunken and in bad condition, it is surmised that it is an effect of letting the alcohol get too weak and then changing to strong alcohol.

Hine has taken the larvæ while digging in the ground in the vicinity of ponds, from under stones on ditch banks, from the water with dip-nets, and occasionally in most unexpected places. He says, however:

"If one is looking for them he is likely to meet with more or less disappointment, as the finding of one specimen does not indicate necessarily that others may be taken under the same conditions. The fact that specimens have been taken from floating logs and débris suggests that they may be transported for longer or shorter distances in this way, and during high water be stranded upon ground which, when the flood subsides, is high and dry and far removed from the bed of the stream. Since the species in all its habits is closely associated with water and wet ground, this seems to be the only way of explaining the appearance of larvæ in dry soil and in places remote from where the eggs are laid."

Hine's description of the larva follows, being in many ways more brief and accurate than that of Walsh given previously.

"Full grown larva nearly 2 inches in length. General color yellowish white, with wide dark brown bands at the union of each two segments. Prothoracic segment on each side with two lateral grooves, which do not quite reach the

posterior border of the segment, and a dorsal (?) groove continued for the entire length. These grooves and a number of irregular dots on the posterior part are dark colored, while the remainder of the segment is light. Mesothoracic segment, on each side, with four longitudinal grooves, which reach nearly the entire length. The dark markings on this segment include a narrow anterior border, the lateral grooves, and a number of irregular dots near the posterior margin. The metathoracic segment is like the last except that the dark color on the anterior margin is wider and the posterior, instead of being dotted, is uniformly brown. The abdominal segments are each similar to the metathoracic, but the dark markings in the region of the lateral grooves are more or less abbreviated. Last abdominal segment with two pairs of dark markings; the ventral pair extend the whole length of the segment and are connected just behind the anal prominence by a cross-band; the dorsal pair are oblong, somewhat irregular in outline, and extend from the anterior margin to beyond the middle of the length. At the anterior ventral border of each of the first seven abdominal segments is a transverse series of prolegs, three on either side of the midventral line. These prolegs are located within the dark transverse bands, but are lighter in color than these and prominent enough to be seen easily. Above the prolegs on either side of the middorsal line is a small swelling which appears as a rudimentary proleg; before the two is a distinct transverse light spot still within the dark area."

"The head of the larva is very small for so large an insect and the mouth parts are minute. The mandibles consist of two strongly chitinized pieces, and work by being pushed endwise backward and forward. When drawn in, the anterior ends point directly forward, but when protruded, these same ends point downward and backward, thus forming a pair of hooks by means of which the prey is held. The larva is able to protrude its mandibles very quickly and to use them very effectively on soft-bodied invertebrates on which it is known to feed."

W. A. Riley and Johannsen (1915) have also figured the full grown larva of *Tabanus atratus* (Plate 3, Fig. 45), and Malloch (1917) figures the head and first segment of the larva (Plate 5, Fig. 77).

On the pupal stage we have information, in addition to Walsh's description, by C. V. Riley, Hart, and Hine.

Riley reports that his larva transformed to pupa within the ground during the early part of July; it remained in this state but a few days and the fly issued July 13, and soon made its presence known by its loud buzzing inside the jar. It was a perfect specimen, and the pupal integument was sufficiently firm and polished so that by carefully washing off the earth an excellent cabinet specimen was obtained, which retained almost the exact form and appearance of a living pupa. Before the escape of the fly, which was effected through

a longitudinal fissure on the back of the head and thorax, reminding one of the mode of escape of the harvest flies (*Cicadæ*), this pupa by means of the horns with which it is furnished had pushed itself up to the surface of the earth.

The pupa itself (Plate 12, Fig. 140) is, according to Riley, nearly an inch and a quarter in length and a third of an inch in diameter. It is cylindric, slightly curved, as in the figure, rounded at the head and tapering at the extreme hind portion. The abdominal segments are, all but the first one, provided with a ring of fine yellow bristles, pointed backwards. There is a stout thorn at the anal extremity, bearing six other thorns.

The pupa state lasts but a few days and before the emergence of the fly the pupa is pushed to the surface of the ground by means of the bristles and thorns of the abdomen, with bending movements of the body.

It splits along the dorsal line and the fly emerges, leaving the pupa case in perfect condition.

Hart's description of the pupa (Plate 13, Fig. 163) contains some additional details.

"*Pupa, Male.*—Length 30–35 mm., diameter 7.5 mm. Yellowish fuscous with a brownish tint, thorax not paler. Palpal sheaths distinct, short, very narrowly separated by a depressed space. Abdomen roughly wrinkled and subopaque. Spiny fringes tipped and annulated with black. Otherwise as in the pupa (female) of *T. stygius*" (described by Hart in the same paper).

Hine (1906) gives another description of the pupa:

"Pupa [Plate 11, Fig. 124; Plate 13, Fig. 164] about 1¼ inches in length. Color brownish yellow. Antennal and other tubercles of the head darker than the surrounding parts. Prothoracic spiracle slightly elevated, clear brown in color, reniform and oblique, rima gradually curved to near the dorsal end, where a distinct hook is formed by a sharp bend. Abdominal spiracles nearly round; rima of the first short and gradually curved and with a slight hook at the dorsal end. Terminal teeth [Plate 13, Fig. 164] arranged in pairs, a ventral pair and a pair on each side formed by a dorsal and a lateral tooth. The distances between these teeth are variable; the two dorsal are nearest together, then follows the distance between a dorsal and a lateral, the distance between the two ventral, while the distance between a ventral and a lateral on each side is greatest of any."

This appears to be all that is known about the early stages of *Tabanus atratus*.

Tabanus atrimanus Loew.—An African species, common near Mt. Mlanje in southern Nyasaland, having a preference for the neighborhood of wooded streams, and being there most abundant in November and December.

Neave collected and bred the larvæ. On October 25 a number of larvæ were taken in the Ruo River among the roots of grasses in running water, but occasional individuals were also found in the mud in other wooded streams. Imagos, bred from these, began to emerge on November 25. At the same spot in the Ruo River some other larvæ were found which may be those of *Tabanus pertinens*, but they were not bred to the adult stage.

The larvæ, which are figured (Plate 3, Fig. 52) are strikingly distinct from those of the apparently closely allied *Tabanus variabilis* Lw. They are of a somewhat opaque yellowish color, with rather faint brown pigmented areas. The pseudopodia are well developed, as is the case with other species found in running water, and there are well marked hairs on the syphon. Though invisible in preserved specimens, two pseudopodia of considerable length are present immediately anterior to the anus.

The pupa is a clear orange-yellow color and the aster is remarkable for the erectness and large size of the dorsal pair of hooks, especially in the female. The dorsolateral comb consists of a few widely spread spines.

The larva (Plate 3, Fig. 52), the pupal aster of both sexes, and the dorsolateral comb of the female are figured (Plate 14, Fig. 173, *a*, *b*, *c*).

Tabanus autumnalis Linné.—Scholtz (1850) reports finding the pupæ of this species, which is common in Europe, with those of *Tabanus tropicus* and *Hæmatopota pluvialis* (see also these species), on an excursion in the neighborhood of Breslau in June, 1850, at the edge of a pond covered with *Lemna*, the water of which was completely polluted from manure piles surrounding it. The pupæ, of which the flies emerged after a few days, were found quite near the edge under a thick moist mass of decaying *Lemna*, together with *Stratiomys* and *Syrphus* pupæ.

The larvæ were first found in 1851, by Brauer and Göszy, on the shores and shallow places of the "Wienfluss" (Danube ?); also the pupæ were found. Kollar, 1854, gives only these few remarks on the subject.

Raillet says (quoted from Brumpt): The larva of this species lives in the water; it breathes the air at the surface of the water, like the larvæ of mosquitoes; they may be destroyed likewise by spreading kerosene oil on the swamps where they occur.

It is possible that the larvæ in which Graber discovered the organ named after him were of this species (Paoli).

Surcouf and Ricardo (1909) report on their observation of an egg mass of *Tabanus autumnalis* L. at Lamballe (Côtes-du-Nord) in August, 1907. The eggs formed a dense mass attached to a reed, and the female, turning the head towards the moist ground, remained immobile, making no effort to escape when it was captured. Unfortunately, the observers waited until the following morning to take the plant with its roots, and in the meantime the grass had been cut.

Graber (1882) gives the illustration of a newly hatched larva of *Tabanus*, which is supposed to belong to *Tabanus autumnalis* (Plate 8, Fig. 99). In this larva Graber studied the chordotonal organs, tactile bristles, and other microscopical details (Plate 8, Figs. 99 to 102). These details are discussed in the chapter on Tabanidæ, description of early stages (see also *Tabanus sp. Nos. 10* and *12*, page 179).

Tabanus autumnalis (Plate 8, Figs. 99 to 102; Plate 9, Figs. 103 to 108) is the species in which the organ of Graber was probably discovered for the first time. Krauss (1879) informs us that Brauer, in the spring of 1875, demonstrated in his entomological class the larvæ of *Tabanus autumnalis*, which had been bred from eggs, and called attention to a peculiar and undescribed organ in the abdomen. Drawings of the organ were made by Krauss but not published: according to the latter, they agree so completely with those published by Graber that it is likely that Graber's "Fliegenmade" was in fact the larva of *Tabanus autumnalis*.

Krauss adds the statement that the larvæ of this species, in contrast to tabanids—the larvæ of which live in damp localities, manure, decaying plant material, or in the ground—spend all their develop-

ment in the water, leaving it only for pupation, which takes place at the edge of ponds, in the mud, among plant detritus, etc.

Krauss expresses the opinion that the organ will doubtless also be found in the imago, and in that case the mystery surrounding it may be cleared up.

Von Friedenfels (1880) reports on a larva which he found in the salt lakes of Siebenbürgen. This larva was aquatic and transparent and was first thought to be an annelid. When sent to Brauer in Vienna, it was identified by the latter as a larva of *Tabanus autumnalis*.

Tabanus bicallosus Ricardo.—A smaller species of *Tabanus*, recorded from Pusa, Bengal, and Madras, by Patton and Cragg, to whom we also owe some notes on its early stages.

As in all the small species observed by these authors, the eggs are laid on blades of grass just at the edge of a shallow stream, or on the leaves of the lotus plant at the edges of small ponds, but never over deep water.

The eggs, as in all the smaller species in Madras, are torpedo-shaped, the eggs of this species measuring 1.1 mm. in length and 0.2 mm. in breadth. Figures of the eggs are given (Plate 1, Figs. 12 and 13).

The egg mass figured by Patton and Cragg seems to consist of two layers spread out one over the other, and the whole "moulded into the hollow of the blade right up to the tip" (as in other small Indian tabanids).

The mature larva of *Tabanus bicallosus* is figured by the authors (Plate 4, Fig. 62), but no description is given. On the drawing the eleventh and twelfth segments appear to be completely fused (?), dorsal pseudopods are well developed, which should indicate an aquatic mode of life. There seems to be a color pattern similar to that of the larva of *Tabanus atratus*.

The pupa is likewise figured (Plate 11, Fig. 135), but not described. In addition, figures are given of the abdominal tip of male and female pupæ (Plate 12, Figs. 144 and 145), showing the arrangement of the six terminal teeth. In the male pupa attention is called to the ribbed anal tubercle and the continuous fringe of spines in front of it, as differing from the condition in the female, which has a simple anal tubercle and an interrupted fringe of spines in front of it.

This is the first and only attempt to separate the sexes of tabanids in the pupal stage.

Tabanus biguttatus Wiedemann.—The early stages of this species, which is widely spread throughout Africa, occurring from the Cape to the Egyptian Sudan, have been described by King (1908).

The eggs, which were obtained in the vicinity of Taufikia, Sudan, in the marshes, are deposited in a rounded mass (Plate 1, Fig. 18) on grass and reeds overhanging a pool. One egg mass that was counted contained about 450 eggs. The entire act of oviposition was not timed, but it lasted well over half an hour. When a female is ovipositing, although usually exceedingly shy, the stem on which she is resting may be plucked and carried away or put into a bottle without disturbing her. Having deposited the eggs, she covers the mass with a creamy white secretion which turns black after a short time.

The egg is spindle-shaped, slightly more pointed at one end than at the other, and white in color. Length 2.5 mm. The eggs under observation hatched in about eight days, but possibly under natural conditions, exposed to the sun, the incubation period would be shorter. On hatching, the larvæ fell into the water, swam to the sides, and buried themselves in the mud.

The larvæ can swim only on the surface of the water, and progress either by a telescopic movement or by lashing vigorously from side to side.

Several methods of rearing them were tried. The majority were placed in a large glass vessel containing mud, living grass, and water. Some were put into jars containing only water, others in dishes containing moist sand, others again in vessels containing sand and water so arranged that there was a pool at one end of the vessel and moist sand at the other.

The larvæ in the vessel containing mud, grass, and water did well, but many were devoured by predacious insects—*e.g.*, dragon-fly larvæ—introduced by accident in the mud and water, and others perished owing to the grass dying and fouling the water during transit. Eventually sand and water in Petri dishes were found to be best, as it could be kept clean and the larvæ easily located when wanted.

At first they were fed on tiny crustaceans dredged from rain pools, but during transit, when these could not be obtained, scraps of freshly killed raw meat and congealed blood from the bodies of gorged mosquitoes were substituted. After arriving in Khartoum their diet consisted of earthworms, as a plentiful supply of these could always be procured.

They grew very slowly and at greatly varying rates. Two larvæ hatched from one egg batch on June 11 measured respectively, five weeks later, 4 mm. and 15 mm. Owing to their telescopic nature it was exceedingly difficult to measure them accurately, so the figure must be taken as merely approximate. They did not appear to be cannibalistic in their habits, as several of various sizes were reared in the same dish and sometimes kept short of food, but were never seen to attack each other. When one died, however, its comrades usually devoured it. When not feeding they spent most of their time buried in the sand, with just the tips of their respiratory syphons showing. If the sand was allowed to dry they became very restless, and would make continual efforts to escape from their jars until water was given them again.

Early in August, when they were about eight weeks old, they ceased feeding and were then transferred to jars containing sand to a depth of 6 cm. They descended to the bottom of these jars and were still there when, about six weeks later, the author went to England on leave.

On January 28 King returned to Khartoum, and it was found that the jars then contained several dead adults—all males—a few dead pupæ and larvæ, and a single live larva. This last died early in February without having reached the pupal stage. The empty pupal cases were all sticking up out of the sand, the pupæ having evidently worked their way up from the bottom of the jars by means of their abdominal spines. In several instances the old larval skin had remained attached to the caudal teeth of the pupal case.

Neave (1915) also found the larvæ of this species in the valley of the lower Shire and its tributary, the Mwanza, in southern Nyasaland. Here, in the sand and mud on the banks of the rivers, large numbers of larvæ were obtained, of which the majority belonged to this species, and these were chiefly found in mud among the *Phragmites*

reeds on the banks and in the back-waters, etc., of the Shire River. They often occurred, especially if the mud was inclined to be dry, at a depth of as much as 6 or 8 inches. The locality figured by Neave represents a large flat sand bank partly covered with *Phragmites*, at the border of a river about 50 feet in width.

The lower part of the Mwanza River was dried up at the time of Neave's visit, except for a few isolated pools, on the banks of which many larvæ of *Tabanus biguttatus* were found in the mud. Many others were subsequently found in the Ruo valley in November.

The larva is, according to Neave, of a type which has many representatives, being of a clear white color, except in individuals about to pupate, with well defined pigmented bands and spots between the segments and on the anal segment (Plate 5, Fig. 69). Several species have larvæ of this type, only varying in the amount of pigment and in the distribution of it on the anal segment.

King's description of larva and pupa follows:

The larva (Plate 5, Fig. 75, *a*, *b*), when nearly hatched, is white in color, but later assumes a grayish to yellowish tinge.

Mandibles (Plate 5, Fig. 78) black, slightly serrated and with two tufts of curved hairs at the base.

First thoracic segment anteriorly brown. Laterally placed on the second and third thoracic segments are brown comb-like marks with the four teeth pointing backwards.

On the anterior portion of each abdominal segment—with the exception of the eighth—are two brown annuli, or rings, encircling the body. The former of these two rings is usually covered by the posterior margin of the preceding segment. The hinder ring bears a double line of fine black hairs and also a number of small fleshy projections or pseudopods. The eighth abdominal segment serves the purpose of a respiratory syphon. Its posterior margin is brown and from its extremity can be extruded a small process terminating in stigmata. A brown curved longitudinal mark is situated on either side of the eighth segment. The anus is placed at the base of this segment. Length 35 mm.

The pupal case is chestnut-brown in color, with the thoracic tubercles darker. Each of these tubercles bears a spine. The abdominal segments are apically ringed with backward projecting spines. The anal segment terminates in a cluster of six teeth (Plate 12, Fig. 148), the dorsal pair larger than the lateral and ventral pairs. Length 20 mm.

The pupal aster has been figured by Neave (Plate 14, Fig. 175, *a–d*); it is of the regular type and the dorsolateral combs have

long spines, which are, however, shorter and stouter (especially in the female) than those of *Tabanus corax*.

Tabanus bovinus Linné.—This is one of the commonest species of the genus in Europe, the largest species in Sweden. It has a wide distribution and occurs even in South Africa.

The larvæ were found by Degeer (1760) in the soil of a meadow. Placed in earth which was renewed from time to time some of them gave pupæ. I give Degeer's original report in translation from the German.

The larvæ of these flies, which have not been recorded by any one before me live in the ground, and I have described them already in the Swedish Proceedings.[16] When in the month of May, I turned over the earth of a meadow, I found many of them, and having placed seven or eight in a jar with fresh earth which was renewed from time to time, I was aware, on June 12, 1760, that one of them had turned to a pupa, and had in part crawled out from the earth, being still fixed in it by the abdomen. I dug for the remaining larvæ, but found only three of them, which pupated later, and in the same way protruded with half of their body from the earth. At last I found one more small dead larva. I presume that those which pupated have eaten up the others.

The largest of these larvæ (Plate 4, Fig. 59, *a*, *c*) was about $1\frac{1}{2}$ inches long, and in the middle $3\frac{1}{2}$ lines in diameter (as seen in the figure), very similar to the larvæ of the large crane-flies. The body was cylindric, of almost equal diameter all along its course, narrowed and pointed at the head, twelve-segmented, the last segment small and wart-like.

Color whitish gray and yellowish; a number of black transverse bands formed by the transverse ridges and some transverse stripes in the incisions between the segments. The small head is shining brown. Under the lens the skin appears also shining, and covered with very fine longitudinal ridges.

The head is elongate, horny, bearing several parts difficult to distinguish as they are constantly in motion, also with two small short antennæ, some pointed parts placed below, and two large horny black mandibles placed above the latter, as long as the head and curved downwards. If a larva was held between the fingers, it exserted the hooks, attached itself to the skin with them, and pulled the contracted body up. Probably it burrows its way in the earth by means of these hooks. While resting, the head is withdrawn into the first segment, and this in turn into the second segment, so that the anterior part becomes of the same diameter as the rest of the body. The two last segments can also be retracted into the third last one, in a similar way.

[16] Bromsarne's Ursprung.

The last segment (Plate 4, Fig. 59, *c*) has the aspect of a small soft textured conical wart; at its end there is a small, elongate protruded horn-like brownish yellow piece placed vertically, which I assume to be a spiracle, as a longitudinal fissure is discernible. Under the segment before the last, near the segment preceding it, there is a fleshy prominence, also with a longitudinal fissure in the middle, the anus.

On the body seven prominent blackish ridges are found situated anteriorly on the fourth, fifth, and the following segments, to the tenth, including the latter. At the sides and below fleshy warts take the place of feet. In contracting the rings the interior parts are drawn in.

The nymph (Plate 4, Fig. 59, *b*, *d*), 1 inch in length, of the same thickness as the larva, cylindric excepting the last segment, which is much smaller. It transformed into a large horse-fly while I had expected that a large crane-fly would appear. The color was brownish gray; darker at the abdomen and the other parts. The abdomen was eight-segmented; at the posterior margin of each segment was a fringe of long gray hairs. On the last segment at the tip there are six hard horn-like points (Plate 4, Fig. 59, *d*), by means of which the nymph works its way out of the earth. Head, thorax, and wing cases, or the whole thoracic region together shorter than the rest. Anteriorly, on the head, there are two small brown tubercles, each of them bearing a hair, probably spiracles. At the side of each tubercle an elongate and equally brownish point, appressed flatly to the head, directed backwards and jointed in the middle, probably the antennal sheaths. If the nymph is touched, it carries out worm-like movements with the abdomen.

Early in July the fly hatched out. The pupal skin splits open dorsally alongside the thoracic shield, and at the sides of the head. Internally, there are in the cuticle of the head two long horn-like spines, the function of which is unknown to me.

These observations of Degeer are remarkable for their precision. A number of details which have been incorrectly represented in tabanid larvæ by several later authors, as the number of segments, position of the anus, etc., have been correctly described. Nothing essential is omitted. Degeer, in 1760, calls attention, like del Guercio in 1913, to the similarity between certain tabanid larvæ and those of crane-flies, but while the latter is satisfied to state that there is no appreciable difference between them, Degeer's description of the *Tabanus* larva remains fully characteristic of the group and probably also of the species, though before the larvæ hatched, he had expected them to become tipulids.

Westwood (1840) has briefly translated Degeer's description, and I give his summary to complement the above rather imperfect translation:

"The larva of *Tabanus bovinus* Degeer (L) is found in the earth, and is of an elongated subcylindric form, attentuated at each end, especially in front; it is destitute of feet, twelve-jointed, having the head distinct, narrow, elongate, horny, armed with two strong curved hooks, antennæ, and palpi; the fourth to the tenth segments having an elevated dorsal papillose ridge used in progression; the terminal segment is minute and tuberculiform; the pupa is naked, incomplete, elongated, subcylindric, with six spines at the end of the body; the margins of the abdominal segments ciliated, and the forehead bi-tubercled."

Westwood also reproduced Degeer's figures, though very imperfectly; also Macquart, 1834.

No further observations have been published on the early stages of this species, since Degeer.

Tabanus bromius Linné.—The larvæ of *Tabanus bromius* live, according to Beling, by preference in the grass-covered soil of meadows, fields, and similar places. They are often brought to the surface by moles, and in the spring and summer, 1874, Beling found them, as also later on the pupæ, especially frequently on meadows in fresh mole hills. The pupal stage lasts usually between two and three weeks, and the imagos begin to appear in the second half of the month of June. The larvæ feed by sucking the contents of earthworms, larvæ and pupæ of other insects, and, if there is lack of other food, of their own kind, but they seem to be able to subsist in case of necessity on earth alone, in which, as Beling says, he kept them for months until pupation.

I assume that the larvæ, if they subsist on earth, will in fact subsist on its organic contents in a similar way, as is the case with earthworms. Moreover, small earthworms and insect larvæ are easily overlooked by the observer if the earth used is not specially sterilized.

Larva.—Length up to 16 mm., width 4 mm., color pale yellow, strong, silky, shining, with distinct dense and fine longitudinal striation, with twelve body segments, excepting the small narrow brown head, which can be retracted into the first segment. The latter in the middle of dorsal surface with a wall-like longitudinal prominence, which is covered in its anterior part with short hairs, and on

each side is provided with a rounded densely brush-like denticulate tubercle. Antennæ not discernible, palpi very short, one-jointed, thick. First body segment short, obtusely cone-shaped, retractile, and merging into the second segment without distinct border. Last segment short and much narrower than all the preceding, cupola-shaped, bearing on the anterior part of the under side the more or less prominent half circular anus crossed by a deep longitudinal furrow or fissure. The tip of the last segment a roundish, wart-shaped, retractile prominence, bearing the vertical stigmatal fissure. Segments five to ten inclusive with marginal swellings anteriorly. In this ridge or ring eight protuberances are distinctly observable, of which two smaller ones are located on the dorsal side, one on each side, and two pairs, placed near to one another, forming the locomotory swellings of the under side. The surface of the retracted head capsule visible through the cuticle of the first segments appears as an extensive brownish yellowish area limited by two broad blackish stripes which, diverging from the middle, are pointed and drawn out behind.

The pupa is likewise described by Beling, a translation of which follows.

Pupa.—Pupa 16 to 18 mm. in length, 3.6 to 4 mm. in width, obtuse at the anterior end, slightly narrowed behind, of dirty greenish yellow color, smooth, somewhat shining, and if older, with blackish borders of the segments. Above the front five to six small flat brownish tubercles or warts, of which the two lowest are enlarged and tooth-like, the two upper ones flatly truncated, bearing a short blackish brown hair in the middle of the truncation. On each side of these frontal tubercles is a short tooth-like outwardly directed spine. Above the frontal tubercles are two small wart-like tubercles each bearing a short black bristle. Lower ventral side of thorax on each side with two small brown warts bearing a backwardly directed closely appressed hair, and with an elongate black spot, these together forming two rows diverging posteriorly. Abdomen nine-segmented, first segment very short, third to eighth segments inclusive on each side with two parallel longitudinal depressions rather distant from one another, together forming two depressed lines running laterally alongside the abdomen. In the middle of these depressions on each of the six segments mentioned and in its anterior part a short spine-shaped prominence and a similar prominence also on the second segment immediately behind the wing cases. Third to eighth abdominal segments near its posterior margin with a circle of densely placed yellowish to brownish or even blackish hairs of uneven length and more or less completely appressed backwards to the surface. Last abdominal segment with six short thick outwardly diverging spines with dark brown tips, of which four are placed in a horizontal row; namely, a strong one on each side and two weaker ones in the middle. Wing and leg cases not very strongly marked, of the same color as the rest of the body, reaching to the anterior border of the third abdominal segment.

The pupa of *Tabanus bromius* L. has been figured also by Surcouf and Ricardo (1909); the previous observations by Beling have not been observed by these authors.

This pupa (Plate 12, Fig. 151) was collected in the earth of a railroad embankment at Longny, Orne, France, by M. E. Cordier, from whom the authors received it. The adult died in the act of hatching; it proved to be a male of *Tabanus bromius* L.; having left the pupal shell half way, it had been unable to free itself in spite of numerous efforts as proved by the pupal shell being unusually drawn out in length. The manner of the hatching of the adult is evident from the examination of this rare piece; the head causes the top of the shell to burst and, consequently, to break open longitudinally, under the pressure of the insect, down to the base of the first abdominal segment, which remains intact like the following segments. When the adult has reached this stage of its development, it takes flight, and goes in search of food as soon as it is dry.

Tabanus carolinensis Macquart.—Of this North American species, Malloch has given us, in his synoptic table, the following data on the pupal stage:

"*Pupa*.—Dorsal abdominal segments, except first, armed with an irregular transverse series, or two such series, of very stout thorns, their bases very much dilated, slightly caudad of which series there are sometimes a few widely separated, much longer spines. Seventh dorsal abdominal segment with 10 or 12 moderately long thorns in a continuous transverse series, slightly cephalad of which is a transverse series of very stout thorns longitudinally in line with the spaces between the thorns of the posterior series."

No illustrations are given.

Tabanus corax Loew.—A large species with wings uniformly dusky to the edge of the apex. Habitat, Africa. According to Neave, common on the southern side of Mt. Mlanje, southern Nyasaland, in the more wooded areas within the belt of heavy rainfall. On the wing from the end of November to the beginning of January.

We are indebted to Neave (1915) for a knowledge of its life history.

The flies were kept by Neave in a comparatively large cage made of mosquito netting and wood, each partition measuring about 5 by 4 by 3 feet, in which were boxes containing grasses and growing plants.

The mosquito netting was loosely attached to the cage, so that the shock to a fly in striking against it was minimized.

One or two captured females of *Tabanus corax* oviposited in these cages, the process in one instance taking nearly an hour, between 3 and 4 p.m. Many egg masses of this species were also obtained in the bush, always on reeds or grasses overhanging mud. While the female is ovipositing she is not easily disturbed, as in the case of *Tabanus biguttatus* (King), and on one occasion one of Neave's collectors brought him the reed, fly and all, from more than a mile distant without disturbing the female. The egg mass with its cement covering is pure white when first laid, becoming dark gray as it hardens. The cement which covers these egg masses must be, according to Neave, water-proof and insoluble, as some individuals from them succeeded in hatching even though the egg mass had been kept in 70 per cent alcohol for two days.

The egg masses of *Tabanus corax* are of the usual tabanid type, all the individual spindle-shaped eggs being laid with their long axes in the same direction. In the cases observed hatching took place about the fifth day, but this would be likely to be lengthened or shortened in some cases, according to the temperature. The process of hatching takes place very suddenly. The egg mass splits in the midline, following the long axis, and the small larvæ emerge almost simultaneously, forming a large quasi-viscous drop which falls bodily from the reeds, etc., into the water or mud below.

A number of newly hatched larvæ were obtained from collected egg masses, generally found on reeds overhanging swampy ground. The young larvæ grow very slowly at first. Neave figures the syphon and anal segment of one of these young larvæ. The figure (Plate 10, Fig. 120) shows the peculiar Graber's organ, somewhat tongue-shaped or triangular, and, according to Neave, attached by fine strands of muscle from each of the three corners, apparently to the body wall. It lies above the gut immediately below the dorsal integument and seems to be capable of motion independent of the general body movements. The organ contains a number of pairs of small black pyriform bodies.

Neave obtained the larvæ of *Tabanus corax* in considerable numbers. Adult specimens are large, from 40 to 45 mm. in length, and

distinct, having a thick rough integument of a dull reddish color, with a tendency to two more definite patches of darker red on the dorsal portions of the last two segments. The general coloration appears to be largely due to the presence of foreign bodies in the rough skin. The syphon is very short, as seen in the figure (Plate 5, Fig. 67).

These larvæ were most ferocious cannibals in all stages and very troublesome in the laboratory, as they seemed to have unlimited power of wandering about, even over dry surfaces. They frequently succeeded in reaching the receptacles in which other species were kept, and in destroying the larvæ in them.

In the pupal aster (Plate 15, Fig. 181, *a*, *b*, *c*) the dorsal hooks of the male are somewhat larger than those of the female. The dorsolateral comb is large and composed of very long and fine spines. The pupal asters of male and female, and the dorsolateral comb of the female are figured.

Tabanus cordiger Meigen.—A species described by Meigen (1820), and widely spread in Europe, chiefly in the South.

Brauer (1883) is the first to figure the larva of this species (Plate 6, Fig. 88, *a–d*), but gives no description.

Picard and le Blanc report that they found, on March 4, 1913, in the trunk of a poplar at the edge of the Mousson River, near Montpellier, France, an elongate, whitish larva, pointed at both ends and having a ridge, or prominent ring, on each segment, a larva which appeared to them to be that of a tabanid. The larva was placed in a jar containing pieces of decaying wood from the poplar tree in which it had been found, and was left without any other food.

The exact time of pupation was not observed, but in the meantime it seemed justifiable to assume that the larva was satisfied with the vegetable food which it obtained from the decaying wood, since one month after its capture it had not yet transformed. On June 10, a male *Tabanus* was observed in the room, which had just hatched, and was determined by Dr. Villeneuve as *Tabanus cordiger* Meig.

The larva lived, at the time of its capture, in the stump of a poplar. The wood of the stump was not yet completely decayed, but rather soft and very moist. A careful search did not reveal any other

larvæ of tabanids or other insects, while the felled trunk enclosed numerous larvæ of Tipulidæ, Asilidæ (Eryx), and some larvæ of beetles (Lamellicornia).

Picard and le Blanc's observations are insufficient for an exact morphological description of the larva, but a brief description of the pupa is given, based on the pupal shell left after the hatching. A translation of this description follows:

Pupa.—(Plate 12, Fig. 136.) Slightly curved inwards ventrally in its posterior part; measures 20 mm. in length, 4 mm. in width, and 3 mm. in height. The anterior part (Plate 13, Fig. 155), extending to the scutellar segment, and comprising the head, thorax, legs, and wings, is smooth and unarmed, except for some dorsal hairs. The posterior part of the pupa (Plate 13, Fig. 158) is composed of seven segments, surrounded at about the second third from their base with a crown of stiff and unequal bristles. The tubercles described by Surcouf in an unidentified pupa were not observed. The last segment is terminated by two three-toothed tubercles, at the base of which is found ventrally a cup-shaped formation which possibly corresponds to the anus of the larva. The preabdominal segment and the first six abdominal segments bear on each side a spiracle placed towards the base of the segment, at the height of the anterior third. The crown of bristles of the seventh segment is incomplete and interrupted dorsally.

The emergence of the adult causes at the anterior part of the pupal shell a fissure which separates it into three wings, two dorsolateral ones and a ventral one of irregular shape. The latter, in the shape of an elongate hexagon of 4 mm. length by 3 mm. width, shows a characteristic ornamentation which can be traced on the figure given.

At the anterior part two tubercles are found, each bearing a bristle; somewhat below two slight ridges which meet in the median line, further down two doubled tubercles, placed between two triangular thickenings, finally, more posteriorly, two pairs of small tubercles between which two depressions with inner convexities are found. A little in front of the point of attachment of the piece, two lateral triangular expansions are observed. It is probable that the first two tubercles correspond to the antennæ of the larva and the others to the mouth-parts.

These observations show, according to Picard, that certain species of tabanids may have wood-inhabiting larvæ, and that their habits are more variable than one would suppose. Picard's paper is accompanied by three figures.

The observations made by Paoli on Graber's organ (page 37) have possibly been made on this species.

Tabanus costalis Wiedemann.—Habitat, eastern North America. The species is very common, and a pest to stock. Of its early stages we know little. There are no data available on its oviposition, and although Hine (1903), while speaking of the control of tabanids in Louisiana, mentions that oviposition in tabanids often takes place not over water but over damp ground, and that "one finds the eggs of *costalis* and a number of other species in such places quite frequently," he gives no description of the eggs.

The larva seems, according to Hart, to be normally a terrestrial larva. He has found it two or three times in the earth of corn fields in Champaign County, Illinois. The dates given are May 31 and June 4. Specimens were placed in a breeding cage, and an imago of *costalis* was secured from them.[17] Hart believes that the species is single-brooded. His description of larva and pupa follows:

"*Larva*.—Length 20 mm., diameter 2.7 mm. Prothorax with lateral shining areas about as long as the dorsal, coarsely striate, a smooth spot near center of disk; dorsal and ventral areas of thorax smooth, a few striæ on those of metathorax, especially posteriorly; remaining areas moderately striate, lateral areas of abdomen a little more finely striate than the others; all more or less shining."

"Dark annuli pale, narrow, longitudinal stripes scarcely present; false feet with dull pubescent crests, their sides rather finely striate; a narrow dark annulus at base of respiratory tube, another around base of last segment, enclosing anal prominence and giving off a pair of lateral stripes, the lower one longer; no projecting spine seen."

"*Pupa* (from defective cast skin of male).—Length 20 mm., diameter 3 mm. Light fuscous brown, shining; abdomen smoothly wrinkled, slightly opaque; prothoracic spiracular tubercles slightly but nearly equally elevated, free margin rounded at tip, rima not vertical, evenly arcuate, slightly hooked in front."

"Abdominal spiracular tubercles small, subtriangular, narrower behind, obliquely subconical, much shorter than basal diameter, bearing a very small subcircular rima; fringes formed of unequal pale spines, the longer ones sparse on seventh segment above; outer terminal teeth twice as large as lower pair, directed laterally and slightly backwards; upper pair smallest, directed upwards; ventral fringe of last segment not noticeably webbed; lateral tufts rather high, not near ends of ventral fringe."

Hart's material was, as he says, "not in the best condition for accurate comparisons."

[17] I have since found the larva of this species in the muddy banks of Lake Carnegie in Princeton, N. J., which shows that it is not always terrestrial.

Tabanus desertus Walker.—South America, British Guiana. The larval and pupal stages have been observed by Bodkin and Cleare in the coast region of British Guiana. Numbers of larvæ were found in a damp accumulation of sweepings situated at the end of a drain leading from a large cattle pen. Several of these larvæ were secured and kept in the laboratory under frequent observation. Although supplied with a quantity of suitable food the largest larva eventually consumed its companions and pupated. After sixteen days a female *Tabanus desertus* emerged. The pupal aster (terminal end of the pupa, showing the arrangement of the spines) is figured (Plate 15, Fig. 180, *a*, *b*).

Tabanus ditæniatus Macquart.—The distribution of this tabanid as given by Austen is wide. In Africa it occurs from the Transvaal in the South to Egypt in the North, while outside the bounds of Africa it is found in Baluchistan, India, Ceylon, China, and Japan. In the Anglo-Egyptian Sudan it occurs fairly commonly in the South, but until 1910 it had not been recorded from the northern provinces, when King began his studies on this species.

The larvæ were taken early in March, 1910, in a small water channel, locally known as a "Gadwal," on the estate belonging to the Sudan Plantation Syndicate Ltd., at Zeidab, Berber Province. The water was for the most part overgrown with a covering of green slime, and when this was cleared away a few larvæ could generally be seen on the surface. On stirring up the mud at the bottom and edges of the water more appear, while if one waited for an hour or so, specimens would continue to rise. They were apparently living in the mud at the bottom of the pools and coming periodically to the surface to breathe. They could be seen rising to the surface by a lashing motion, and if left undisturbed would after a few seconds sink out of sight again.

Some forty odd larvæ of various sizes were taken on March 9 and placed in a jar containing water, slime, and hollow grass stems; most of these had disappeared by the next morning, the larger ones having devoured the smaller ones. On March 10 more than a hundred were obtained, and, together with the survivors from the previous day, were divided among three jars (only three being available), two con-

taining wet mud, and the third water with hollow grass stems and other debris. Earthworms were provided as food, but were not taken very readily; the larvæ seemed to prefer to eat each other. They were brought to Khartoum on March 11, and the following morning each of the thirty-three which were still living was placed in a separate jar containing clean river sand and water. They fed freely on tiny earthworms, but their numbers steadily decreased until about April 16, when thirteen survivors, having attained maturity, ceased to feed. Up to this stage, if the sand in which they were living was allowed partially to dry out, they became very restless until water was given them again, but hereafter they preferred sand which was only slightly damp. In appearance as well as habit they altered considerably at this stage of their existence. While young and growing, they possessed well developed prolegs and conspicuous dark dorsal markings; now, however, their prolegs became small, and in color they appeared uniform yellowish white.

These thirteen larvæ were left undisturbed until May 26, when one specimen was washed and was found to have pupated, probably within the previous two days, as the eyes had not begun to show the color which they acquired later. On the following day, two more pupæ were discovered in the sand. Prior to pupating the larvæ had made a number of tunnels in the sand, and the pupæ were lying in a more or less upright position in the tunnels and near the surface.

On April 28 King left Khartoum, and traveled in the provinces until May 30, by which date one larva had died and twelve completed their life cycles, producing eight females and four males. The first had emerged on April 29 or 30, so the period passed in the pupal stage was probably about six days.

King's detailed description is given in about the following words.

Immature Larva.—(Plate 3, Fig. 47; Plate 4, Fig. 57, *a–d.*) Length 18 mm. Color yellowish white, with dark markings composed of pubescence. Mandibles dark brown to black, slightly serrated. Anterior margins of the meso- and metathoracic segments dark, except on the venter. A ring of pseudopods, eight in each ring, two dorsal, two lateral, four ventral, on the anterior third of each abdominal segment except the eighth, well developed, except the dorsal pairs on the first and second segments, and bearing spines or hooks. Spines are also present between the pseudopods on each ring. The rings on the first and

second segments edged before and behind with dark pubescence, especially on the dorsum, the pubescence extending between the dorsal and lateral pseudopods, thus enclosing the dorsal pseudopods in a dark ring. On each of the third to the seventh segments inclusive is a patch of dark pubescence between the lateral and the dorsal, and between the dorsal pseudopods, three patches on each ring, the median patch being conspicuous. To the naked eye these median patches constitute a median dorsal line of black dots.

On each of the third to the sixth segments, inclusive, are two patches of dark pubescence, immediately anterior to the dorsal pseudopods. The posterior margin of the eighth segment bears dark pubescence. The surface of the larva other than that bearing pubescence is shiny and longitudinally striated.

Mature Larva.—(Plate 4, Fig. 57, *b*; Fig. 64, *a*, *b*, *c*.) Length 25 mm. Color yellowish white. Mandibles dark brown to black, slightly serrated. Thoracic segments shining and longitudinally striated, except the anterior margins, which are opaque and pubescent. On the prothoracic segment are five longitudinal grooves, one ventral, two sublateral, two subdorsal, not extending to the posterior border. On the meso- and metathoracic segments are eight such grooves, four on either side. The first abdominal segment bears one pair of ventral pseudopods;[18] the second segment one pair of ventral and one pair of lateral; the third to the seventh, two pairs of ventral and one pair of lateral. Traces of most of the other pseudopods are present, especially of the dorsal pseudopods on the fourth to the seventh segments. The pseudopods bear small colorless spines or hooks, and similar though smaller spines are situated between the pseudopods and on the dorsum of the first, second, and third segments where the pseudopods are wanting. On the dorsum of the first and second segments these spines constitute a double band. The posterior third of each abdominal segment is shiny and longitudinally striated. The anus is edged with pubescence. The syphon when exserted appears rather shorter than the eighth segment.

Pupal Case.—(Plate 11, Fig. 129; Plate 12, Fig. 141.) Length 17 mm. Color yellowish brown, thoracic tubercles and abdominal spiracles darker, the former bearing hairs. On the posterior third of the second to the seventh abdominal segments is a ring of backwardly pointed spines, shortest on the second segment and longest on the seventh. The eighth segment (Plate 12, Fig. 141, *a*, *b*) terminates in a coronet of six teeth, chestnut brown in color, darker at the tips, the lateral pair by far the largest, the dorsal and ventral pairs being about equal in size, the former sometimes slightly the larger. The dorsal pair arises from between the lateral teeth, the four teeth constituting a row. Ventrally placed to this coronet are two rows of similar teeth, each row consisting of from two to five teeth, the two rows together constituting an interrupted transverse row. These teeth are unequal, and vary in size and number in different specimens.

[18] King uses the term "pseudopod" instead of "proleg" which should be used, pseudopod being used in protozoology.

The pupa, when first formed, is yellow with a greenish tinge, especially on the thorax. Later, as the imago develops, the eyes show as deep maroon and the thorax becomes generally darker.

On *Tabanus ditæniatus* we possess also some notes by Patton and Cragg, who observed the species in Madras (1913).

Tabanus ditæniatus, being a small species, oviposits, according to these authors, "on blades of grass just at the edge of a shallow stream, or on the leaves of the lotus plant at the edges of small ponds, but never over deep water." It should then fall under the heading of those tabanids in which the larvæ are said to have no air sacs and to die when falling into deep water.

The eggs of *Tabanus ditæniatus* measure, according to Patton and Cragg, about 1.2 mm. in length, and 2 mm. in breadth, being slightly more slender than those of *Tabanus bicallosus* Ric., an Indian species, studied by Patton and Cragg (1913).

The egg is figured by Patton and Cragg (Plate 1, Fig. 17), evidently showing a dark band placed subapically, the extreme tip being also dark on the figure. The egg mass of the same species is figured as spread out in a single layer on a blade of grass (Plate 2, Fig. 21).

The mature larva is also figured (Plate 4, Fig. 61); attention is called to the short stout syphon tube.

The pupa is figured by the same authors (Plate 11, Fig. 133), and the eighth abdominal segment of the pupa is figured (Plate 12, Fig. 147) to show the arrangement of the terminal teeth and the anus.

With these observations our knowledge of the species is quite complete.

Tabanus epistates Osten Sacken.—Of this North American species, Malloch (1917) has given us, in his synoptic table, the following data on the pupal stage:

"*Pupa*.—Dorsal abdominal segments, except first, armed with an irregular transverse series, or two such series, of very stout thorns, their bases very much dilated, slightly caudad of which series there are sometimes a few widely separated much longer spines. Seventh dorsal abdominal segment with the posterior transverse series consisting of two long, widely separated spines on the middle portion, and several, closely placed, on each lateral extremity which are but little caudad of the much shorter thorns of the anterior series. The portion

of head-capsule between bases of antennæ slightly elevated, rounded, fairly rugose, and not carinate or divided below; abdominal armature moderately strong, distinctly biserial laterally."

No illustrations are given.

Tabanus fraternus Macquart.—An African species, but uncommon in so damp and well wooded a locality as Mt. Mlanje, where a single female was bred by Neave on December 16, 1914, from a locally collected larva, which was not recognized as distinct from that of *Tabanus tæniola*.

The pupal aster (Plate 15, Fig. 182, *a*, *b*) resembles that of *Tabanus maculatissimus* in having a small papilla on each side of the midline. The dorsolateral comb consists of only a small number of spines, which though rather long are fairly stout. The pupal aster and dorsolateral comb of the female are illustrated.

Tabanus fronto Osten Sacken.—This American species has been recorded from North and South Carolina, Texas, and Florida. We know nothing of its egg-laying habits, but the species has been bred repeatedly from larvæ, by Brimley, in Raleigh, North Carolina. The larvæ of this species occurred freely in the soil in Brimley's garden, in a comparatively dry locality situated on the crest between two water sheds, the nearest permanent water being at least a quarter of a mile away. These larvæ are described as white with pale brown transverse bands, and transform into pupæ in June or July, and into flies some two or three weeks later. The earliest date on which an adult emerged was July 4, which is also the earliest date on which Brimley has seen the species in the fields. Two larvæ, which had been preserved in alcohol, were yellower than the *trimaculatus* larva described by Brimley, but showed no trace of the pale brown bands which exist in life. The largest of the two measured 36 mm. long, and was taken July 5, while the smaller one was 33 mm.. taken on March 31. Both, as also the preserved *trimaculatus* (?) larva, were well, but not abnormally, extended.

The only pupa reported of this species was found by Brimley under a stone in his back yard.

Although horse-flies do not generally breed away from water, *Tabanus fronto* seems to be an exception, as larvæ have been taken in

Brimley's garden in several different years, while the adults occur more commonly in the garden and house than any other species of the family, the flies quite frequently entering the house, while newly emerged specimens have been noted on a number of occasions.

Tabanus (*Atylotus*)[19] *fulvus* Meigen.—A European species. Sharp describes a larva (Plate 4, Fig. 60, *a–d*) which he says may belong to this species. This description follows:

"In a larva, probably of this family (Tabanidæ), found by the writer in the shingle of a shallow stream in the New Forest (England), the annuli are replaced by seven circles of prominent pseudopods,[20] on the abdominal segments, about eight in each circle, and each of these feet is surmounted by a crown of small hooks, so that there are fifty or sixty feet distributed equally over the middle part of the body without reference to upper or lower surface. The figures of the larva of *T. cordiger*, by Brauer, and of *Hæmatopota pluvialis*, by Perris, are something like this but have no setæ on the pseudopods."

Tabanus fuscipes Ricardo.—An African species reported by Neave from Lake Chilwa, southern Nyasaland, in January, 1914, in circumstances which render it extremely probable that they had bred in mud some distance from water, which had been hard and dry for some portion at any rate of the dry season. Neave is inclined to think that in this and other mid season species, such as *Tabanus claritibialis* Ric. and *Tabanus sandersoni* Aust., the larvæ hibernate fully fed at the beginning of the dry season and only pupate when the next season's rains release them from the hard ground.

Tabanus glaucopis Meigen.—A European species, occurring in Austria as well as in Scandinavia, but evidently rare. According to Brauer, the larvæ have been observed by Wahlberg (1838) in noctuid caterpillars. I was not able to find the statement quoted by Brauer, but it may be correct, as Wahlberg made numerous observations on parasitic and semiparasitic dipterous larvæ.

In as far as most of the Bombyliidæ are truly parasitic, and tabanid larvæ often burrow deeply into their prey, it is not unlikely that tabanids belonging to the Pangoniinæ which are apparently related

[19] Sharp (Insects, p. 483) uses the generic name *Atylotus*, which is a subgenus of *Tabanus*.

[20] Sharp, like King, uses pseudopod for proleg.

to the Bombyliidæ, should be of more pronounced parasitic habits. In *Tabanus glaucopis* we have probably to deal with an occasional parasitism, not typical for the species.

Tabanus gratus Loew.—An African species, and, according to Neave, a very common one and one of the earliest on the wing in southern Nyasaland, occurring sometimes in August.

Larvæ and pupæ were collected in this month, the first individual emerging in the laboratory on September 1. The larva (Plate 3, Fig. 51) is moderately pigmented, though compared with that of *Tabanus insignis* the pigmented areas are nearly confined to the edges of the segments and are not nearly so dark in color. The syphon is somewhat longer than in that species. A few larvæ were obtained in the stream beds near Mt. Mlanje and a small series in Portuguese territory to the east of the mountain early in October.

The pupal aster (Plate 15, Fig. 186, *a–d*) is of the normal type, being regular in outline. The spines of the dorsolateral comb are much reduced, especially in the male.

Tabanus hilaris Walker.—A species of Bengal, Assam, and South India, on the early stages of which we have some notes by Patton and Cragg (1913). According to these authors its habits of oviposition are the same as in *Tabanus striatus*, that is, it oviposits as a general rule on blades of grass, pieces of stick, etc., at the edge of a river, stream, or pond. The eggs when deposited are brownish white. No figures or descriptions are given.

Tabanus ignotus Rossi.—A species not listed in Schiner's Fauna Austriaca, and, according to del Guercio, synonymous with *Tabanus albipes* Fabr. Del Guercio has given a somewhat unsatisfactory account of its life history, and its early stages. The species has appeared, according to del Guercio, in extraordinary numbers, in company with tipulids, in the rice fields of the region of Bologna (Italy), and the larva is said by him to have caused considerable damage to the rice fields.

The species, of which del Guercio furnishes a brief description, not stating, however, on whose authority the insects were identified, appeared in the region (Molinella) from the first ten to twenty days

of June. The flies are found on plants like *Arum*, *Sagittaria*, *Typha*, etc., on which they move about frequently with a buzzing sound.

The eggs are deposited on the leaves of the plants mentioned and others, forming circular crusts, which were seen abundantly after oviposition had taken place, that is, during the whole month of July, while some belated specimens may oviposit as late as August. A more detailed description of egg or egg cluster is not given.

Larvæ were never found in the fall, but half grown larvæ were found in winter, most of them being ready to transform in April or May of the following spring.

Without insisting on the strangeness of the fact, del Guercio reports that these larvæ are so similar to those of the tipulids found by him (*Tipula oleracea* L.) that without an accurate examination it would not be possible to distinguish them. They are more robust, more cylindric, but of the same color; they are in the same way provided with brushes of hairs on the segments of the body, but the mouth-parts have mandibles of about twice the size and thickness of those of the tipulids, from which they differ only by the kind of velvety combs found on the clypeus and in the whole anterior part of the head. The figure to which del Guercio refers, is given (Plate 7, Fig. 96, *a*, *b*) for comparison. The habits of these larvæ are said to be the same as those of the tipulids with which they are found, and to behave in the same way when transforming into pupæ.

I have given this description as it is the purpose of the present report to give a full account of all that is known about the early stages of Tabanidæ. But it may be permissible to express a reasonable doubt whether del Guercio did not eventually figure two tipulid larvæ, having taken one of them, erroneously, to be a *Tabanus*. As no reference is made to any previous descriptions of tabanid larvæ, excepting (see below) *Tabanus autumnalis*, and in this case also the literature is not quoted, and as there is no mention of the structures common to all known tabanid larvæ, as the prolegs, syphon, respiratory tube, serrated mandibles, the assertion that the larva of this *Tabanus* can hardly be distinguished from that of *Tipula oleracea* cannot be taken seriously.

The transformation takes place, according to del Guercio, from the end of May to early June, when the larvæ leave the rice fields in large numbers for their metamorphosis in the dams and meadows.

While the *Tipula* appears in two generations, the life cycle of the *Tabanus* comprises only one generation, which begins at the end of spring of one year and is completed towards the middle or end of spring of the following year.

The larva of *Tipula* and those of *Tabanus* are, as del Guercio insists, of the same dark gray color as the soil in which they live and resemble one another not only in shape but also in many of their organs, including the mouth-parts, in such a way that there remains no point of resemblance, even in this regard, between the larva of the *Tabanus* of the rice field and that, for instance, of *Tabanus autumnalis*, the larvæ of which differ from it in every detail.

The pupa, however, is figured by del Guercio (Plate 11, Fig. 123) as the pupa of this *Tabanus*, for comparison with the tipulid pupa (Plate 11, Fig. 122), and is in fact in every way a real *Tabanus* pupa, except that on the figure no spiracles are visible, which were apparently overlooked by the author in making the drawing.

Del Guercio's statement that the damage done by tipulids and tabanids in the rice fields resulted in the destruction of the whole cultivation, has probably to be corrected in that as far as any larvæ, the tipulids may possibly have been responsible for the damage; for the tabanids, however, this is very unlikely, and, as long as del Guercio has, in fact, made no observations whatever on the food of his larvæ, we are bound to assume that the *Tabanus* larvæ, which were apparently numerous, but of which we are still awaiting description, fed on the larvæ of *Tipula oleracea*, and were in this instance certainly not injurious.

I wish here to call attention to the fact that Degeer has already spoken of a great resemblance between tabanid and tipulid larvæ; in fact, before he knew that the larvæ found by him were larvæ of *Tabanus*, he expected crane-flies to hatch. However, his description, the first one ever given of a *Tabanus* larva, establishes beyond doubt the characteristics of these larvæ by which they differ from those of tipulids.

Tabanus insignis Loew.—(*Tabanus sharpei* Aust.). An African species, common in southern Nyasaland, near Mt. Mlanje, from November to March.

The very characteristic and strikingly pigmented larva (Plate 3, Fig. 53) was, according to Neave, common in the mud of the forested streams from the end of September. It may be distinguished at a glance from other similarly pigmented species by the white trefoil-shaped area on the dorsum of the anal segment. This is a voracious and predacious larva and troublesome to keep in the laboratory for that reason.

The pupal aster is of the normal type, the spines of the dorsolateral comb being few in number but somewhat long. A small series of adults was bred by Neave from the larvæ above described. A certain number of these flies belonged to the type of *Tabanus sharpei* Aust., and the two forms, the larvæ of which are identical, are connected by a great variety of intermediates.

The larva is figured (Plate 3, Fig. 53), also the pupal aster of the male and the dorsolateral comb of both sexes (Plate 15, Fig. 184, *a*, *b*, *c*). In the figure, the larva looks much like that of *Tabanus atratus* but the lateral stripes are poorly developed except on the thoracic and on the ninth and tenth segments, while on the eleventh segment they are more completely fused with the transverse ones into a broad pigmented band.

Tabanus kingi Austen.—*Tabanus kingi*, recorded from Khor Arbat, Sudan, Africa, is a species superficially resembling *Tabanus tæniola*, and allied to species of *Tabanus* from Abyssinia at present undescribed. The life history has been worked out by King (1910), who observed the species in Khor Arbat, in a locality consisting of a stream of slightly brackish water running in a gorge on a rocky hill. On emerging from the hills into the plain the stream disappears in the sand. In the autumn, during the brief rainy season, it comes down in sudden overflow, and is then of considerable size, but in April, the month in which these observations were made, it is, except where there are pools, not more than a few inches in depth. The bed of the stream is stony and there is little or no vegetation on its banks.

The female fly deposits her eggs in a rounded mass on a rock rising sheer from the water generally slightly overhanging, and from six to fifteen inches above water level. Rocks chosen for this purpose overhang comparatively deep pools, from eighteen

inches upwards, in which the water moves but slowly. Such rocks occur only here and there, in the mile or so of stream searched, only three rocks bearing traces of having been used by this tabanid for purposes of oviposition being found. On one of them were the remains of several hundred egg masses lining a small crack in the face of the rock from 2 to $3\frac{1}{2}$ feet above the water level. As none of the fresh egg masses found were situated more than 15 inches above water level, these old masses had probably been deposited when that level was higher. Altogether seven females were taken in the act of ovipositing, and several more were seen. No particular time of the day seems to be chosen for the act; one was found ovipositing at 11.40 a.m. and another at 4.40 p.m., and unlike *Tabanus biguttatus* Wied., the only other horse-fly King has observed ovipositing in the field, this tabanid does not lose her natural wariness while engaged in depositing her eggs. In fact, she is often more difficult to capture then than when merely sunning herself on a rock.

The egg masses, figured by King (Plate 1, Fig. 20), vary in size, and no count of the number of eggs contained was made, but the average mass is believed to consist of about 500. When freshly laid the mass is glistening white and can be seen from a considerable distance, but within a few hours it takes on a mottled gray hue which so closely resembles the color of the rock that it is not easily detected. While the fly is occupied in laying her eggs, numbers of a tiny Hymenoptera assemble and proceed to add their eggs to the mass, continuing to do so after the fly has gone. From some twenty egg masses collected from the rocks about equal numbers of this egg parasite and of the tabanid larva were obtained. Specimens of these Hymenoptera were sent for identification to the Scientific Secretary of the Entomological Research Committee. They proved to be a new species of Chalcidæ, and have been described by Mr. J. C. Crawford, of Washington, under the name of *Telenomus kingi* (footnote to King's paper, by Mr. Guy A. K. Marshall).

One horse-fly[21] taken in the act of ovipositing completed her egg laying in a collecting box on the evening of April 13. These eggs had

[21] King frequently uses the Arabic word "seroot," for horse-fly, by which the flies are known in the Sudan.

hatched by the morning of April 19, the incubation period being therefore about five days. Under normal conditions, exposed to the sun, it may possibly be less. The larvæ from these eggs were allowed to fall from the egg mass into a basin containing water and stones and were provided with portions of earthworms, and tiny coleopterous and dipterous larvæ obtained from wet moss. They refused to feed, however, and all died; probably at this stage of their existence they require brackish running water.

In places the stream at Khor Arbat is very shallow and ripples over and around stones; under these stones larvæ of various sizes, mostly nearly mature, were taken. Apparently stones which were not quite or were barely covered with water were chosen by the larvæ in order that they might come up to breathe without losing their hold and so be in danger of being carried away by the current. Usually only a single larva was found under one stone, and, in every instance where two or three were together, a mortal combat was taking place. If a larva was placed on one's hand it would at once endeavor to drive its mouth hooks through the skin, and where the skin was thin, it would succeed in inflicting a sharp pricking pain. Owing to these cannibalistic habits the number of larvæ which could be transported was restricted to the number of vessels available, so, though nearly two hundred were taken from the stream, only forty-two were brought alive to Khartoum. There they were placed in jars containing coarse sand, brought from Khor Arbat, and water, and fed on medium sized earthworms. They took these willingly when hungry but appeared to need food only every two or three days. King left Khartoum on April 28, Captain W. B. Fry taking care of the larvæ during his absence, and on King's return on May 30, the majority of these larvæ were dead. One, however, had completed its life cycle and seven were still living. Six of these seven pupated during the next few weeks but died as pupæ. The pupal period is probably about six days, for one which pupated on May 5 appeared to be mature on May 11, when it perished.

One empty pupal case was taken under a stone in the bed of the Khor Arbat stream; the fly must have crept up the stone through several inches of running water before gaining the air.

Although this tabanid, according to King, in its adult form closely resembles *Tabanus tæniola* Pal. de Beauv., in its larval stage it differs markedly from that species. The larva is admirably adapted for clinging to stones in rapidly running water, its usually long pseudopods with strong hooks, being retractile and capable of being used as suckers. None of the other tabanid larvæ observed by King possessed an anal proleg.

Besides the seven specimens mentioned above as having been taken in the act of ovipositing, two more were caught sucking blood from camels. No males were seen.

King describes the early stages in about the following words.

Egg.—Length 2 mm. Color white, becoming darker as the embryo within develops. Spindle-shaped.

Mature Larva.—(Plate 4, Fig. 56 and Fig. 65, *a*, *b*, *c*.) Length 35 mm. Color pale gray to dusky gray to deep chestnut-brown. Mandibles dark brown to black, long and powerful, slightly serrated. Anterior margins of meso- and metathoracic segments dark. A smooth shiny pale area on the dorsum of each thoracic segment; on the prothorax this area is concave anteriorly, convex posteriorly, and with parallel sides. On the meso- and metathoracic segments it appears to the naked eye diamond-shaped, though sometimes it is actually hexagonal. On the venter of the prothorax are two shiny pale longitudinal areas, each bearing several long black hairs arising from a single pore; a similar but larger area is striated on each of the meso- and metathoracic segments, bearing two similar tufts of hair. On either side of the meso- and metathoracic segments are three longitudinal areas not extending to the margins of the segments, longitudinally and deeply striated. On the anterior margins of the meso- and metathoracic segments on either side are four paler lines extending backwards, to form the divisions and edges of the three striated areas. On the anterior third of each abdominal segment except the eighth is a ring of pseudopods, eight in each ring, two dorsal, two lateral, four ventral, except on the first segment, where the dorsal pair is wanting. The dorsal pseudopods are never well developed, and, with the exception of those on the fifth, sixth, and seventh segments, unprovided with hooks. The lateral and ventral pseudopods are very long and bear at the apices long, strong hooks, chestnut-brown in color, sometimes darker at the tips. On the median pair of ventral pseudopods on the fourth, fifth, and sixth segments, these hooks form a complete circle, but on the remaining pseudopods bearing hooks the circle is incomplete. Immediately below these hooks is a row of tiny spines. Immediately behind the ventral pseudopods on the first to the seventh segments inclusive is a shiny striated area. On the venter of the eighth segment, anteriorly placed to the anus, is a pseudopod equal in size and similar to the ventral pseudopods on the other segments, and bearing an incom-

plete circle of hooks. Scattered over the surface of the larva are occasional black hairs. The syphon, when exserted, is shorter than the eighth segment, and bears a number of black hairs. The dark appearance of the larva is due to tiny dots of pubescence arranged closely together, except on the shiny areas mentioned above.

The skin of the larva frequently bears scars of old wounds.

Pupal Case.—(Plate 12, Fig. 137 *a, b, c.*) Length 20 mm. Color yellowish brown, thoracic tubercles and abdominal spiracles darker, the former bearing hairs. On the posterior third of the second to the seventh abdominal segments inclusive is a ring of backwardly pointing spines, shortest on the second segment and longest on the seventh. The eighth segment terminates in a coronet of six teeth, chestnut-brown in color, darker at the tips, the lateral pair by far the largest, the dorsal and ventral pairs being equal in size. These teeth are arranged roughly in a circle. Ventrally placed to this coronet are two rows of five comparatively thin spines, of varying length, together constituting an interrupted transverse row. Dorsolaterally placed to the coronet are two rows of spines similar to the ventral row.

The dorsum of the abdomen is sometimes clothed with black pubescence arranged in four longitudinal stripes. On the sixth and seventh segments these stripes merge and on the seventh segment the pubescence is confined to the posterior third. The pubescence is wanting on the dorsum of the eighth segment but is present on the venter of the seventh and a small patch is situated immediately below the coronet on the eighth segment.

The pupa when first formed is yellowish. Later, as the imago develops, the eyes show as dark spots with a greenish tinge and the thorax becomes generally darker.

Tabanus lasiophthalmus Macquart.—This species, which is widely spread and occurs, according to Hine, in eastern North America, Columbia, and Chile, has been reared by Hine (1906) from the egg to the adult. The fly is one of the earliest of the genus to appear in the spring, adults having been taken at Columbus, Ohio, as early as May 20, and it is common during the first half of June. The eggs are placed in masses on various plants that grow in low, wet ground, but Hine has not observed them over water. The masses are shiny black when fully colored, rather small for members of the genus, only slightly convex, and accompanied with an unusual amount of cementing material, which nearly obscures the form and arrangement of the individual eggs. The mass suggests somewhat a drop of tar or other black substance fastened to the surface of a leaf of the common cattail reed (*Typha latifolia*), a sedge, or some other plant.

The eggs are usually deposited after June 10, and the specimens from which larvæ for rearing hatched were taken in Medina County, Ohio, on a common sedge found growing near the outlet of a small spring. They were collected June 28 and hatched the next day and the day after. As Hine had not been successful up to this time in keeping very young larvæ for any length of time, it was decided to try different methods of treatment in order to find out, if possible, that which is best suited to their requirements. Some were placed in a jar containing water only (No. 1); others in a jar containing water with a couple of inches of sand in the bottom (No. 2). A third jar (No. 3), in which larvæ were placed, contained wet muck, while the fourth lot (No. 4) was placed in a jar containing moist sand to the depth of about 3 inches, covered over the top with a quantity of fine leaves of water plants. In all the breeding jars were placed plenty of small crustaceans and other minute invertebrates procured from water by means of a finely-meshed sieve.

It was soon observed that the larvæ in breeding jar No. 4 fed on the crustaceans, and at the end of a few days showed a distinct increase in size. Those in the jars containing water soon died, and jar No. 3 did not appear to be a success, so all but No. 4 were abandoned. The larvæ in this last, however, were separated, and placed in similar jars, one specimen in each, and reared to full size, the adult fly being procured the following spring.

Since, as stated, three of the four jars started were soon abandoned, what is said hereafter regarding the method used in rearing pertains to the single one retained. A glass jar was selected so that the actions of the larvæ could be observed; a small jar seemed desirable because the larvæ are predacious and eat their own kind as readily as anything else, for which reason it is necessary after a short time to place only a single specimen in a jar; also, even a small receptacle furnishes plenty of room and the long series which it is desirable to have takes as much space in the insectary as one cares to give to a single species. Only the quantity of sand and other material necessary to success should be placed in the breeding jar, as it is desirable once in a while to look this material over carefully in order to locate the very small specimens and find out what they are doing.

Half pint jelly glasses were found by Hine to be well suited for the purpose and easily obtainable. Covers proved to be desirable in order to prevent too rapid evaporation of moisture, but a small perforation or two in them was necessary to furnish ventilation. As the muck which was tested as soil for the jars grew much mold, clean lake sand was chosen as decidedly preferable for the purpose. The covering of plant material mentioned furnished a resting place for the small crustaceans offered for food, and the larvæ themselves seemed to choose to remain in it in preference to burrowing into the sand, although they were apt to be found in any part of the jar. Algæ made good material for covering, but only a small amount could be used, and too much water was detrimental as either in excess tended to cause decay, and consequently a bad odor, which was observed to be unfavorable to the insects. The principal point in favor of the algæ was that they contained no hollow stems or large pieces into which the larvæ could crawl, but still, being composed of small soft particles, furnished a mat in which they could hide. When it was desired to locate these larvæ it was easily done by picking the mass to pieces. As odors, which are often fatal to the larvæ, were likely to develop from the material put in for food and also from other sources, it was found necessary to watch the jars continually, giving them a thorough cleansing once in a while, and perhaps putting in fresh sand and plant material occasionally.

Larvæ when first hatched were about 2 mm. in length; they grew rather slowly, but in fifteen days after hatching had doubled their length. They fed readily on the small crustaceans which were given them. It was impossible to give these small crustaceans their proper surroundings, so many of them died, and it was observed that the young larvæ fed on these as well as on the specimens which they killed themselves. The larvæ could be seen crawling about in the jars; they appeared to remain very near the upper surface of the sand most of the time, and when food was scarce did much crawling, but when food was plentiful satisfied their appetites and hid among the plant material where they remained quiet.

A difference in size in the various larvæ soon became apparent, and the older they became the greater was this difference. On July 23, twenty-five days after hatching, some specimens measured as much

as 7 mm., while others measured only 3 mm. At this date angleworms were given for food, and were accepted readily, and appeared to be as satisfactory as the crustaceans, but it would seem that the latter are preferable for the stage just after hatching.

On July 27 some of the larvæ were 10 mm. in length, and on August 2 the same specimens measured 12 mm.; thus at this stage they grew more rapidly than when they were younger. They fed actively till about the middle of September, when they had become apparently full grown, or 25 mm. long. Length in the larvæ of tabanids is, according to Hine, not a satisfactory means of indicating the size, for the segments telescope on one another in such a way that it is difficult to take two measurements exactly alike, but an endeavor was made in this case to make the different measurements similar, so I believe that those given are considered sufficient to indicate the comparative sizes of the different ages. After September 15 the few specimens remaining alive buried themselves in the sand of the breeding jars and were quiet most of the time until March 10, when one pupated, the adult emerging on the 25th of the same month. The others died before the pupal stage was reached. Hine has noted that larvæ of various species of tabanids taken from their natural habitats during the winter did not produce adults in the spring much before the same species appeared naturally, but in this case, where the specimens was kept under artificial conditions during its entire life, the adults appeared almost two months earlier than is normal in nature.

Hine's description of the larva follows:

"*The mature larva* [Plate 3, Fig. 44] is not notably different from those of other species of *Tabanus* so far as form and appearance are concerned. The color is a dirty white with a pinkish shade over most of the body; the prolegs are not so prominent as in many species, and on this account specimens appear somewhat maggot-like. On either side of the body is a longitudinal row of very small black spots or specks, one to each segment and located just above the ventral prolegs; these spots are lacking on some of the anterior and some of the posterior segments; their presence appears to be characteristic of the species, at least so far as my acquaintance with different larvæ goes. Mature specimens are about 25 mm. in length."

Hine has not taken the larva of this species in its natural habitat, therefore he cannot say where it is to be found, but he thinks that

it lives in debris, or in the ground around low places near where the eggs are laid.[22]

"*The pupa* [Plate 11, Fig. 127] is somewhat dusky in coloration, the thorax being almost black. The terminal teeth of the abdomen [Plate 13, Fig. 159] are quite different from those of any species studied so far, and these differences alone make its determination easy. The dorsal and lateral teeth are much larger than the ventral, the lateral being much larger than any of the others; the ventral teeth point almost directly backward, while the direction of the others is largely upward. The thoracic spiracle is rather small and nearly longitudinal, its rima is curved, but no distinct hook is formed at the posterior end. Length 18 mm."

We owe our knowledge of the early stages of *Tabanus lasiophthalmus* entirely to Hine.

Tabanus laverani Surcouf.—An African species, being rare in Neave's locality, Mt. Mlanje in southern Nyasaland, where only occasional specimens were taken.

A single female was bred on November 25, 1913, from a larva collected near Neave's headquarters. The larva did not belong to the pigmented type like that of *Tabanus gratus* and resembled the larva of *Tabanus variabilis* in bearing lateral prolegs on the anal segment. It was, however, of a yellower color and less transparent than that species, and lacked any pigmentation on the syphon.

The pupal aster (Plate 15, Fig. 185, *a*) is remarkable for the great size and elongation horizontally of the middle pair of hooks. The dorsolateral comb is reduced to two very short processes, the main combs on the last segment being also of this character, as may be seen from the view in profile.

The pupal aster and dorsolateral comb of the female are figured (Plate 15, Fig. 185, *a*, *b*, *c*).

Tabanus lineola Fabricius.—A common species, inhabiting eastern North America, common in states as wide apart as Massachusetts, Ohio, New Jersey, and Louisiana.

We owe to Hart a description of larva and pupa. The larva closely resembles the young of *nigrescens*, and was not separated from it at first. Examples were taken at Hart's collecting station, C, near the

[22] I have since found (May, 1917) the larva of this species in the muddy bank of a rapid flowing brook in the neighborhood of Princeton, N. J.

foot of Quiver Lake, Illinois, over sand, mud, and algæ vegetation; at Station I, in the bed of the slough with grass, rushes, and willows, and a very shallow stream of spring water when the river is low; and at Station H, on the Illinois River below Havana, Illinois, where it is narrow, (the east bank steep and sandy, a layer of mud over sand at lower levels, the water quickly deepening, considerable current, a little vegetation; on the west bank mud, steeply sloping, trees but almost no vegetation, decided current), on April 14, 15, and 30; and in Flag Lake, (shallow, muddy, bordered with rushes, thick with floating vegetation), on April 27—as shown by specimens preserved. The larvæ were also taken April 8 and June 15 and 24 in Sand Lake, Lake County, Illinois, and in ponds in Kane and Champaign Counties.

I give Hart's description of the larva.

"*Larva.*—Length 20 mm., diameter 2.7 mm. Prothorax with lateral shining areas about as long as the dorsal area,[23] striation about the same as that of the upper mesothoracic area, no noticeable central smooth spot, a small one on the lower margin posteriorly; remaining lateral areas a little more finely and closely striate; dorsal and ventral areas of thorax nearly smooth on disk, with basal striæ; those of abdomen with moderately close striæ, more or less interrupted on disk; all areas more or less shining."

"Surface whitish, dull pubescent markings very light brown but distinct, annuli narrow, crests of false feet also dull pubescent, their sides striate; lateral stripes of thorax distinct, slender, not dilated posteriorly, lateral edges of dorsal areas of thorax diverging. An opaque dark ring about base of respiratory tube, and another encircling anal prominence, above it usually three light brown spots."

"Main internal tracheæ rather thick and noticeable, subparallel, not strongly sinuate, at least back of the middle. Terminal stigmatal spine often protruded."

Of *Tabanus lineola* Hart has obtained three pupæ on May 18 of different years. Imagos were obtained from these on May 27, 29, and June 6. The tabanid pupæ develop, according to Hart, much more rapidly in hot weather than in cold, and to this fact is probably due the difference in time of emergence. Another pupa was taken at Matanzas Lake on August 24. Hart's description of the pupa follows:

"*Pupa.*—[Plate 12, Fig. 149; Plate 13, Fig. 162.] Length 19 mm., diameter 3 mm. Light ferruginous brown, shining, abdomen roughly wrinkled and sub-

[23] I cannot confirm this character from material collected in Princeton, Spring, 1917.

opaque. Palpal sheaths indistinct, not distant; tubercles not dark; ocellar tubercles indistinct or wanting; thoracic spiracular tubercles slightly but nearly equally elevated, free margin rounded at tip, rima not vertical, evenly arcuate, slightly hooked in front."

"Abdominal spiracular tubercles subtriangular, narrower behind, obliquely subconical, much shorter than basal diameter, bearing a small subcircular or short and strongly arcuate rima; on anterior slope a transverse groove, usually longer than the rima; fringes formed of unequal pale spines, only one or two long spines above on seventh segment; outer terminal teeth much longer than the others, directed laterally and upwards, the tips of the four upper teeth about in line. Fringe anterior to anal prominence showing a chitinous webbing between the bases of the spines, so that the separated tufts of the female look like a pair of broad low teeth with several spiny points; lateral tufts low down, near ends of ventral fringe, formed of short spines."

The pupa is also figured by Malloch (from Hart's material), (Plate 12, Fig. 149).

Oviposition and eggs are not known. Also it remains to be determined whether there are one or two broods of this species in one year. Adults were taken as early as May 17, and as late as September 27, with dates of capture in June, July, and August.

Tabanus maculatissimus Macquart.—An African species common in the neighborhood of Mt. Mlanje, southern Nyasaland, where Neave obtained data on the early stages.

The larvæ were found in mud in a partially dried up stream, and from these a few individuals of the adult were bred during November. The larvæ were obtained in Portuguese territory to the east of Mt. Mlanje. These larvæ were not, however, at the time distinguished from those of *Tabanus biguttatus*, of which they were thought to be immature examples. The figure (Plate 5, Fig. 71) is from other individuals, obtained subsequently, which are believed by Neave to belong to this species. This figure shows the pigmentation pattern of the eleventh segment considerably developed, while the dark circular band at the posterior end of the segment is comparatively narrow.

The pupal aster (Plate 14, Fig. 178, *c*) is normal except for a papilla on each side of the middle line, about the middle. There is a well marked dorsolateral comb, consisting of comparatively short stout spines. The pupal aster and dorsolateral comb of the female

and the dorsolateral comb of two different male individuals are figured (Plate 14, Fig. 178, *a–d*).

Tabanus medionotatus Austen.—An African species, to which Neave attributes, with some doubt, a series of specimens collected in southern Nyasaland in 1915. Of this species five males and three females were bred between the end of September and the beginning of November.

The pale colored larva has rather long prolegs, a ring of pigment of varying width around the base of the syphon (Plate 5, Fig. 68), and another ring around the anus, which is usually prominent in the living larvæ. There are also present two prolegs immediately anterior to the anus, but these are not visible in the somewhat contracted preserved specimen and therefore are not shown in the figure. The pupal aster (Plate 14, Fig. 174, *a, b*) resembles that of *Tabanus obscuripes* in having a large and even longer, but less horizontal, middle pair of hooks. Not only the dorsolateral but also most of the lateral comb is absent.

Tabanus melanoceros Wiedemann.—A species recorded from the Atlantic States from New Jersey south.

Late in March, 1909, Brimley in Raleigh, North Carolina, found, while looking under stones in a small clear woodland stream, a *Tabanus* larva which was quite lively and seemed thoroughly at home in clear water. He kept the specimen in a bottle with some wet leaves and practically forgot it. However, on May 18 it had transformed to a pupa, and thirteen days later, on May 31, a male of *Tabanus melanoceros* emerged from the pupa. The larva was approximately the same size as the *trimaculatus* larvæ collected by Brimley, and was like them white without darker bands.

Tabanus nagamiensis Carter.—An African species, only recently described, of which a single female was captured by Neave on the Malosa River, the Anglo-Portuguese boundary south of Mt. Mlanje, southern Nyasaland, on October 8, 1913. A male was bred from a collected pupa on September 27, 1913.

The pupal aster (Plate 15, Fig. 179, *a, b*) somewhat resembles that of *Tabanus laverani* in the great development of the middle pair of

hooks, the dorsolateral comb is absent, a characteristic which Neave has not seen in the pupa of any other species of *Tabanus*, except in *Tabanus medionotatus*. The other combs are, however, striking and characteristic, as may be seen from the figure.

Tabanus nigerrimus Zetterstedt.—Of this European species we have some indication that it is lignicole in its larval stage. Scholtz (1850) expresses the opinion that perhaps some *Tabani* live in their early stages in the detritus of old tree trunks. He found, in July, 1848, in a deep wooded ravine near Charlottenbrunn, on the decaying trunk of a tree of *Fagus sylvatica*, a newly hatched but already fully colored specimen of *Tabanus nigerrimus* Zett., which had previously been found in Sweden and Norway.

Tabanus nigrescens Palisot de Beauvois. –This species is nearly related to *Tabanus stygius*, occurring in the Atlantic States (New Jersey), and also in Illinois. Of its oviposition nothing is known, but Hart has observed the larva and given us a description of the pupa.

An undersized larva, supposed to belong with those of *stygius*, pupated May 18, and on June 1 produced an imago of *Tabanus nigrescens*. Most of the larvæ, treated in Hart's paper as *stygius* were very uniform in size and characteristics; Hart, though unable to separate the two species at this stage, believes that the bulk of his material at least was *stygius*. The imago of *nigrescens* had been taken previously near the Mississippi, in southern Illinois, on August 10.

"*Pupa, Male.*—Length 25 mm., diameter 5.5 mm. Palpal sheaths narrowly separated, about one fourth as far apart as the setæ borne by the large frontal tubercles, a smooth depressed space between them, without tubercles. Lobes of carinate transverse ridges of head more rounded and separated by a deeper notch than usual. Abdomen a little more shining and more smoothly wrinkled. Otherwise not different from the pupa of *stygius* (female) next described."

From Malloch's analytical table the following data are obtained on the pupa:

"*Pupa.*—Dorsal abdominal segments, four to six at least, with one or two transverse series of short irregular spines, the bases of which are not much dilated,

and slightly caudad of these is a transverse series of closely placed, very long, slender bristles. Length at least 25 mm.; abdominal spiracles with very much elongated vertical rima, the upper and lower extremities slightly curved forward. The long spines on dorsal abdominal segments either black-tipped or all pale, without a black preapical ring; short spines in anterior dorsal series slender and very uneven. A small but distinct tubercle just in front of base of middle leg in addition to and some distance above the one bearing the paired hairs."

Malloch's material, which was partly the same as that collected by Hart in 1895, contained only one specimen of *nigrescens* (in very poor condition).

The prothoracic spiracle of the pupa has been figured by Malloch (Plate 13, Fig. 156).

Tabanus obscuripes Ricardo.—An African species, occurring in southern Nyasaland, chiefly on the plains in Portuguese territory to the east and south of Mt. Mlanje, in October and November.

A single male was bred by Neave from a pupa collected on October 1. The pupal aster, which is figured (Plate 15, Fig. 183, *a*, *b*) somewhat resembles that of *Tabanus laverani* and has the same large middle pair of hooks. The dorsolateral comb is reduced to a single knob-like process.

Tabanus (*Neotabanus*)[24] *ochrophilus* Lutz.—A common Brazilian species. On its early stages we possess some notes by Lutz in Rio de Janeiro (1914).

Larvæ of this species were found in muddy or sandy soil below and at the sides of a small brook with slowly flowing water. Large quantities of sand had to be sifted to obtain them. As food, tubifex was given. The full grown larvæ measured 30 mm. Color creamy white, integument shining and transparent, intestine reddish or blackish. Tracheæ silvery. Mandibles dark, serrate beneath. Digestion slow, four to five days. Before pupation, contracted; in spontaneous death, expanded.

Culture in moist sand, but for observation purposes moss in glass jars was preferred. The larvæ have to be kept in the dark during the intervals of observation. The pupal stage lasted about ten days.

[24] The few species of *Neotabanus* of which early stages are known are here classified with *Tabanus*. There is no doubt that the genus *Tabanus* could be subdivided into smaller genera or subgenera but opinions on this point are not definite.

The eyes change color after two or three days. Eye coloration and stripes of abdomen visible two to three days before hatching. Pupal shell very transparent.

Tabanus orientis Walker.—A *Tabanus* occurring in North India, Nepal, Bhutan, particularly in high elevations; according to Baldrey, "by far the largest of the species caught at Muktesar (7,500 feet); it is caught at the beginning of March and appears then to be quite full grown (owing to its torn and worn out condition)."

According to Baldrey, who tried to breed the species for experimental purposes, it begins laying its eggs during the last weeks in April, and all flies dissected up to June 19 were found full of eggs.

The eggs when first laid are of a creamish white color, but after a few hours this changes to a cigarette-ash color. They are laid in batches resembling bunches of bananas.

Four lots of eggs were found in jars containing flies; in two cases the only fly in the jars was found dead.

Tabanus par Walker.—Of this African species we possess full descriptions of the egg, larval, and pupal stages by King (1910), who not only secured oviposition in captivity, but also bred the species through from the egg to the adult stage.

Occasional specimens of this tabanid were met with on the White Nile from Gebelein southwards. In the country behind Bor females were abundant and seemed to spend their time resting on foliage, waiting for approaching animals which they would attack at once. No eggs could be found, though a careful search was made in all the places that were considered likely to serve as breeding grounds; hence a number of females, gorged with blood, were placed in a breeding cage, in which was also a dish containing mud, water, and growing grass and weeds. The flies fed on sugar and water, and though the majority died within the first two days, the survivors eventually produced three small batches of eggs (Plate 1, Fig. 10). The eggs obtained in this manner were deposited on May 23 and 24, on the under sides of the leaves of a water weed. Unlike the eggs of most members of the genus *Tabanus*, they were not closely packed in a rounded mass, but placed vertically and separately though in a

cluster. The single egg is "spindle-shaped, about 1.15 mm. in length and white in color, becoming darker as the embryo within develops."

The eggs obtained hatched on May 30, consequently after an incubation period of six to seven days, and the tiny larvæ (Plate 1, Fig. 11) were divided into three lots, and placed in glass basins containing mud, water, and growing grass. These basins for purposes of reference were labelled A, B, and C. At the time when the eggs hatched, King was in the Sudan region, where it was impossible to land and obtain any subterraneous insect larvæ or tiny fresh water crustaceans for them, so they were offered the expressed stomach contents of gorged female ticks —*Rhipicephalus simus* —taken from a dog. A few fed once or twice but the majority refused, and all buried themselves in the mud.

On June 11, the larvæ from A were transferred from mud to clean river sand and water, and given freshly killed mosquito larvæ. They fed on these readily and grew apace, though at greatly varying rates.

The larvæ in B were also given mosquito larvæ from June 11, but they refused to feed, and the mud in which they were living was several times allowed to dry up. On July 11 they were placed in clean river sand and water, and at once began to feed and grow.

On July 19, when King returned to Khartoum, their diet was changed, owing to the difficulty of obtaining mosquito larvæ, to freshly killed and bruised earthworms. They did not take readily to this food, and some died, while others disappeared from the basins. At the time it was thought that they had become cannibals, but eventually it was found that they were being taken by mice. The stock of larvæ from A and B had by this time become reduced to one, which appeared to be full grown and so was killed and preserved.

On July 26 the larvæ from C were transferred to clean river sand and water. It was then fifty-seven days since they had emerged from eggs, and they had spent a great part of that time in a dry cake of mud. Occasionally this mud had been moistened, and food offered them, but they had very rarely taken it. Most of them were alive, but with the exception of a few which were slightly larger than when just hatched, they had not grown at all. They now under more favorable conditions fed readily on a mixed diet of earthworms and mosquito larvæ and grew, some rapidly, others more slowly.

On September 3 and 4 one pupated, lying on the surface of the sand, partly submerged in water, and six days later gave rise to an adult female. By October 18 several more had completed their life cycles, and on that date, as King was proceeding to England on leave, the remaining ones were killed and preserved.

All those that pupated did so on the surface of the sand, some high and dry, others half in and half out of the water. "Probably," says King, "under more natural conditions, the pupal stage would be passed buried in the soil the structure of the pupal case seems to indicate this."

The average pupal period was from six to eight days.

The following are practically King's descriptions of larva and pupa (that of the egg having been quoted previously) of *Tabanus par.*

The mature larva (Plate 3, Fig. 48) when fully extended measures about 13.5 mm. Color white with a grayish tinge. Mandibles dark brown to black, serrated. On the anterior third of each abdominal segment except the eighth is a ring of pseudopods, eight in each ring—two dorsal, two lateral, and four ventral—except on the first abdominal segment, where the two dorsal ones are wanting. On the second abdominal segment the two dorsal pseudopods are very small. The pseudopods are largest on the third, fourth, and fifth abdominal segments, and are always more developed on the ventral than on the dorsal surface. Each pseudopod bears a crown of colorless spines or hooks, and there are patches of dark spines between the pseudopods. The spines on the dorsal sections of the rings on the first and second abdominal segments are dark. The anus is situated ventrally, at the base of the eighth segment, and is fringed with blackish hairs. The syphon tube consists of two segments, and, when exserted is as long as the eighth abdominal segment.

The pupa (Plate 12, Fig. 142, *a*, *b*) is from 12 mm. to 15 mm. in length and at first yellowish white in color, becoming darker as it nears maturity. The eyes show plainly through the pupal case as dark greenish purple. The empty pupal case is yellowish brown, the thoracic tubercles and the spiracles being darker than the surrounding parts. On the apical third of the second abdominal segment is a fine ring of backwardly pointing spines. Similar but broader rings, bearing longer and stronger spines, are on the third, fourth, fifth, and sixth abdominal segments, and one of intermediate breadth on the seventh. The eighth abdominal segment terminates in a coronet of six teeth, in color shining brown, becoming darker at the tip. The dorsal pair are smallest and close together, the ventral pair next in size and wider apart, and the lateral pair longest and arising from almost the same level as the dorsal pair. Ventrally placed to this coronet of teeth are two rows of small teeth, from two to four in each row,

together forming an interrupted transverse row. These teeth are of unequal size and vary in their relative size in different specimens.

Tabanus pertinens Austen.—An African species, according to Neave usually confined to comparatively low-lying country where the river beds are of a sandy nature. In southern Nyasaland it was found common on the Mwanza River, in the Shire Valley, as early as the end of July, but did not occur on Mt. Mlanje, though a few specimens were taken by Neave at some distance from the mountain.

A pigmented larva (Plate 3, Fig. 54), which it was thought might belong to this species, was taken in some numbers in the Shire River in August, and in the Ruo River in October. It was found in both cases in water amongst the roots of grasses or water plants, and seems to prefer rivers with a sandy bottom and banks. The striking larva is remarkable for the development of the dorsal prolegs, which perhaps are associated with its comparatively free-swimming existence. The prolegs are also present immediately anterior to the anus.

Tabanus quatuornotatus (*quadrinotatus*) Meigen.—Common in Europe. This was the first species of tabanid in which the eggs and the act of oviposition became known. The credit of the discovery is due to Joseph Mann, Curator of the Imperial and Royal Zoological Cabinet in Vienna, who, according to Kollar's report in 1854, during a naturalist's trip to Carniola, Austria, in May and June, 1854, succeeded in observing in a damp meadow at Wippach the female of *Tabanus quqtuornotatus* in the act of oviposition. Mann's own words, quoted by Kollar, are translated as follows:

> On June 25 at 11 a.m., I found a *Tabanus* just beginning to deposit eggs on a grass blade; as it did not attempt to escape, I cut off the blade and took it home. Towards 2 p.m. the *Tabanus* flew to the window; I at once looked at the grass blade and found on it a cluster of eggs completed. These were wax-yellow at first, later on they took on a grayish color, and two days afterwards they appeared almost black.

Mann later on found, in the field and also on other plants, several egg masses similar to the first, bringing them all to Vienna.

By this discovery it was known for the first time that the *Tabanus* does not deposit the eggs on the ground where, according to Degeer, the larva lived, but on plants; further, that the *Tabanus* does not

scatter its brood as many other species of flies do, but deposits the eggs in a single mass.

The eggs were counted, and their number found to be about 350 to 400. Also the hatching of young larvæ was observed, and the duration of the egg stage found to be from ten to twelve days (Kollar).

From the egg masses collected by Mann, besides young *Tabanus* larvæ, also ichneumonid flies (parasitic Hymenoptera) were observed to hatch; to Kollar and Mann consequently credit is to be given for the first discovery of hymenopterous egg parasites in tabanids, though no description is given (see under Parasites of the early stages of Tabanidæ, page 182).

The publication of Kollar has been quoted in detail by Lécaillon, to whom we owe further studies on the early stages of *Tabanus quatuornotatus*, chiefly on the oviposition of this species. Lécaillon found the species, with a related form, *Tabanus bromius*, abundantly at Gouy, Aisne, France, in May, 1904. Oviposition of *Tabanus quatuornotatus* was observed on May 24, 1904. In the afternoon of a sunny day, on a wooded and not especially damp hillside, a female was found sitting immobile on a dry branch of a weed (*Origanum*), the head turned downwards, at a distance of 35 to 40 cm. from the ground. The eggs already laid formed a conspicuous white mass. On coming nearer, Lécaillon ascertained that the *Tabanus*, which under every other circumstance is likely to take flight, was not disturbed and continued to oviposit. Lécaillon broke off the branch with the insect on it, placing the whole in a jar. The female continued to oviposit for about ten minutes, when it ceased and left the branch. The egg has been figured by Lécaillon.

Lécaillon calls attention to the fact that, as already stated by Kollar, the ovipositing female is somehow indifferent to what is going on around her, a fact which should be taken into account in understanding the habits of adult tabanid flies.

Concerning the early stages, we learn from Lécaillon's observations on this species that oviposition in some tabanids may take place in comparatively dry and not necessarily in a damp environment. In both Mann's and Hart's observations it took place in a moist environment. As among insects frequently not only the necessities of embryonal but also of larval life may be anticipated from the manner

and conditions of oviposition, Lécaillon thinks that as in tabanids oviposition may take place, according to species, either in a dry or in a damp locality, "not only the embryo but also the larva" can live under very variable conditions of humidity. Lécaillon considers that observations on the larval life of *Tabanus quatuornotatus* verify this hypothesis.

Later, in 1905, again large numbers of egg masses of this species were found by Lécaillon, and his first observations could be generalized to some extent. The eggs were always laid on warm sunny days in early June about the middle of the day, and almost without exception on dried out twigs of various herbaceous plants. In fact, of sixty egg masses collected in 1905, not a single one was found on a green leaf or stem. During the subsequent years, few exceptions to this rule were noticed. In 1907, however, Lécaillon found an egg cluster fastened to a green stem of a grass, and in 1908 a cluster fastened to the dry branch of a tree (bouleau) which had been rammed into the ground. Lécaillon discusses (1906) this fact, putting the question whether we might be dealing with mimetic resemblance. This he thinks hardly probable. The females, avoiding the green objects, would alight only on twigs of grayish or blackish color. More likely it seems to Lécaillon that the females oviposit on dry twigs because these are more rigid than the green stems. In fact, it is observed that during the act of oviposition, the stems of weeds on which the insects alight are curved, especially when agitated by the wind. On the dried out twigs the *Tabanus* is in a much more stable situation and can with greater facility give to the egg mass its rather complicated form.[25]

The places where eggs had been deposited were in all cases examined on woody slopes distant from any water courses. This fact alone is certainly sufficient to prove that the larvæ of *Tabanus quatuornotatus* are not aquatic but terrestrial.

The egg masses are found in large numbers assembled in certain places on hillsides which evidently present all the favorable conditions to which the ovipositing females are adapted. This circumstance is

[25] Lécaillon's explanation is more than doubtful. Many species of tabanids complete their egg masses on quite slender reeds and grasses. Also for *Tabanus quatuornotatus* it remains doubtful by what stimuli it is attracted to the twig.

favorable to the destruction of the eggs in the case of very dangerous species which should be brought under control. The egg masses are in fact very conspicuous and may be collected easily and quickly. All egg masses observed were placed about 30 to 50 cm. above the ground.

The latest date on which oviposition was observed was June 14; however, it may be that it takes place even later, as Lécaillon found eggs containing larvæ as late as July 22 (1907). The period of reproduction extends in this species, at least in certain years, from the end of May to the end of June. The period given in Lécaillon's note in 1906, of two to three weeks, was evidently too short.

A detailed description of the egg mass is given (Plate 2, Figs. 33 to 38). The egg mass presents the aspect of a roughly subconical body which under natural conditions is placed with the base below and the vertex above. The axis of the cone is arranged about parallel to the branch or stem which serves as support, and is placed in such a way as to cut lengthwise through the conical surface and to be partly enveloped by it. However, it would not be accurate to speak of the egg mass as subconical, as certain authors (Brauer and Hart) have done. The body of the mass is distinctly bilaterally symmetrical, as shown in the accompanying figures. The plane of bilateral symmetry is determined by the stem which serves as a support and by a crested longitudinal line placed on the side opposite the egg mass (Plate 2, Figs. 34 and 35). On the other hand, it is possible, with regard to the mass, to distinguish a vertical direction or orientation, a horizontal anteroposterior one, following the plane of symmetry, and a horizontal lateral one, placed at right angles to this plane. In the anteroposterior direction the base of the mass has its greatest dimension (5 mm. in a specimen examined). In the two other directions the dimensions are about the same and somewhat smaller than in the preceding one. Posteriorly, the surface adhering to the support is much larger than the opposite surface anteriorly. Finally, in the specimens examined, the base of the mass was not flat but perceptibly and progressively excavated from the periphery towards the center.

The egg mass is composed of eggs which are placed quite regularly on top of each other, if observed vertically, or placed in successive

horizontal layers, if observed horizontally. The eggs are, moreover, glued together by a substance which hardens after deposition, and causes the eggs to adhere strongly to each other. It goes without saying that the number of eggs contained in a horizontal layer diminishes if we proceed from the base of the mass towards its summit.

The color of the egg mass, which is white at the moment of deposition, is changed rapidly into brown and later into black. The change of coloration begins some time after oviposition is completed; it appears at first at the summit of the mass, thence it spreads towards its base, which after a few hours has turned almost completely dark brown or black. This change of coloration in the egg masses of tabanids has already been observed, as Lécaillon emphasizes, by Mann and also by Hart. However, Mann, who observed the same species as that studied by Lécaillon, speaks of a wax-yellow color which later passes into blackish brown, and Lécaillon assumes that Mann has not noticed the primitive white color of the eggs when first laid, or that he possibly dealt with a different species. Hart's statement that in the eggs of *Chrysops mœrens* (*æstuans*) the first color is cream, seems better to agree with Lécaillon's observation. This may possibly be merely an inexactness of terms, as one author may call an object white which another would call pale yellow or cream.

The change of coloration which takes place in the egg mass a short time after oviposition must be considered as advantageous, as we have to deal with the substitution of a protective color (black) for a white color which as actually observed, renders the eggs very conspicuous to the eye.

According to Lécaillon, the color of insect eggs may be due either to a coloration of the yolk, or to a coloration of the egg envelopes. Changes of coloration can consequently be due to changes taking place inside the egg, or to changes taking place in the envelopes. In the case of *Tabanus quatuornotatus*, the substitution of the white color by a dark color is due to a brown pigment which develops in the chorion after oviposition, probably by the slow effect of air and light. This pigment is very strongly developed and hides completely the contents of the egg. Consequently even at the end of embryonic development when the eggs contained wholly white larvæ, they still are in external appearance completely black.

The pigment is, according to Lécaillon, not altered by alcohol, while in eggs in which the color is due to the yolk the latter is usually decolorized by the action of alcohol.

The female constructs its egg mass, depositing the eggs one after the other, beginning at the anterior end. At last, when the mass has reached a certain width the eggs are found arranged in horizontal and more or less regular layers, while the lower border of the mass forms a pronounced projection, the beginning of the depression of the lower surface. While the female is sitting with head turned downwards the tip of the abdomen is placed upwards, towards the point where the egg is to be laid.

The eggs themselves are of curved shape, they measure about 2.5 mm. in length and only 0.5 mm. in width. Their convex side, which is chiefly visible in those which lie at the periphery of the egg mass, is always directed towards the outer side of the latter.

Lécaillon calls attention to the significant regularity of tabanid egg masses. Among the greater part of insects which deposit their eggs freely on leaves, twigs, walls, etc., the egg mass consists generally of an agglomeration of any shape which it may assume under the conditions presented by the object on which the eggs are laid. In *Tabanus quatuornotatus*, however, the case is different; the egg mass has a very complicated structure, which to a great extent is independent of the shape of the supporting object, and of a strictly bilateral symmetry which can certainly be regarded as a characteristic of evolutionary perfection. From Hart's figures it is seen that the same character is found also in the egg mass of *Tabanus atratus* and *Chrysops æstuans* (*mærens*); and it is probably generally found among tabanids.

In a later paper, in 1911, Lécaillon reports additional observations made on this species in 1906, 1907, and 1908, on the conditions under which the eggs are laid, and a more amplified description of the egg masses themselves.

Since the first publication, a considerable number of egg masses of *Tabanus quatuornotatus* were examined and a number of individual differences were found in the arrangement of the egg masses. Some of the more interesting forms met with were figured in the plate added to the second publication. The egg mass previously described is

consequently incomplete; moreover, they are found frequently in places where this *Tabanus* oviposits. But in many cases the egg masses have been found to be much larger than the one described in 1905. In cases where the egg mass, fastened to a twig, reaches its maximum of development, it is much more prolonged vertically than the one figured in Lécaillon's first report, and it possesses a second plane of symmetry which is horizontal and at right angles to the principal plane of symmetry passing through the stem which serves as support and through the anterior tip which presents the egg mass. But only rarely will the egg mass be found entirely completed in this manner, and all the intermediate forms are found between that described in the first publication and that described later, with two planes of symmetry.

Frequently the egg masses are found grouped together on the same stem, numbering two, three, or four (Plate 2, Figs. 35 to 38).

Finally, as stated also in Lécaillon's short note of 1906, two egg masses are frequently not only contiguous but actually form the continuation of each other (Plate 2, Fig. 33). Of these joint egg masses Lécaillon is uncertain whether they are produced by one female or by two different females. Probably the latter, inasmuch as in the figures given their size is considerable; also from our knowledge of the egg-laying instinct of the female, it does not discern between the eggs laid by itself and eggs laid by another; consequently it will be likely to continue the process of oviposition started by another female in case it, by chance, had alighted on a stem where already other females were laying. As during the process of laying the single egg is always placed beside another previously laid, one would expect that in starting an egg mass, preference would be given to a place where eggs are already present instead of places where only a smooth surface is given.

Sometimes the herbaceous stem which supports the egg mass is very fine, and in these instances the latter surrounds it almost completely, even from behind, following the median line of the stem. In other cases the egg mass is attached to the top of the spike of a grass plant; in this instance it envelops only a small part of the object, so that from behind the egg mass appears as of considerable width.

Besides, the egg masses are never found of a strictly geometrical form, and numerous irregularities in details of their structure are always observed. In general, however, it can be said, that the eggs are placed horizontally and in rows which when observed horizontally or vertically always present a more or less regular arrangement.

On the larval stage of this species we possess the results of Lécaillon's first studies (1905), amplified by later additions. At the time of Lécaillon's studies knowledge about tabanid larvæ was still very meager; he believes that the larvæ are predacious, and there are certain species which are aquatic and others in which the larva lives in the ground or in moist soil.

Lécaillon made an attempt to raise the larvæ of *Tabanus quatuornotatus* from the egg, but did not succeed completely, as many of the larvæ died in spite of all precautions; but he was able to keep them alive for several months and some of them were still living when the paper was published (December 15, 1905), having survived for more than six months. Lécaillon made some observations on the hatching of the larvæ.

According to Mann, the duration of embryonic development in this species is ten to twelve days. Lécaillon placed eggs laid May 24 in a moderately damp atmosphere (the stem with the eggs was simply placed in a crystallizing dish), and observed that on June 6, thirteen days after oviposition, no hatching had taken place. Taking two eggs out of the mass, he saw, as a result of the slight pressure or traction to which they had been submitted, the hatching of completely developed larvæ.

On the following day the egg mass was vigorously rubbed, and as a result all the larvæ hatched at once, showing that embryonic development had been completed in all the eggs. The difference in time appearing in Lécaillon's observation as compared with that of Mann is said to have no significance as the duration of embryonic development is very variable, in a given species, according to the temperature in which the eggs are kept.

The medium duration of embryonic development was, according to Lécaillon's first publication, from twelve to thirteen days. But later he found that the larvæ generally do not hatch as soon as they are completely developed; they may remain inside the egg shells for

a period which seems to be rather long. In 1907, Lécaillon found that eggs which were laid on June 11 had not yet hatched on June 28. Eggs collected on July 11, which had certainly been laid a long time, produced larvæ on July 22. Embryonic development is, however, completely terminated at the end of twelve or thirteen days, because, after that period, it is sufficient to rub the egg mass only slightly in order to cause the larvæ to hatch.

It can be assumed, consequently, that the larvæ do not leave the egg shells in which they have developed, until they need food; from the time of their formation to that of hatching, they probably find an efficient protection inside the egg shells. Perhaps also the egg shell which has to be pierced by the larvæ in the act of hatching is of a variable resistance according to the conditions of the environment. In a damp environment, for instance, the shell would be less resistant than in a dry atmosphere, and the larvæ could hatch much more easily in the first than in the second instance. The larva appears in this case to be adapted to remaining enclosed in the chorion of the egg for a time long enough to wait for favorable atmospheric conditions. Lécaillon has not made exact studies to determine this point.

When the larvæ hatch, they remain sometimes for a few moments on the surface of the egg mass. They carry out very varied movements of contraction and finally fall to the ground into which they burrow immediately. Often they are also attached to one another, forming sort of batches which finally lose hold on the egg mass and fall to the ground. They are, as Lécaillon says in 1906, extremely lucifugous (negative phototropic), and they could not be easily destroyed.

Immediately after hatching, the larvæ are white in color and this color is retained. The body is also somewhat transparent, and the arrangement of different internal organs can be observed with facility. It is seen that in the middle intestine there remains a rather large quantity of nutritive yolk, a remainder of the primitive egg contents. At the level of the yolk the intestinal tract is opaque. When placed in damp earth, the young larvæ appear not to take food for several days.

In order to ascertain whether these larvæ were carnivorous, Lécaillon supplied them with ant nymphæ freed from their cocoons, with flies killed previously, and with larvæ of *Chironomus*. He

found that they did not attack these with great alacrity, but seemed rather to avoid their contact when placed near them. However, after having pierced *Chironomus* larvæ by means of a pointed scalpel, Lécaillon observed some of the larvæ to suck up the blood, their intestinal tract assuming a characteristic red color; he even saw them penetrate into the body of the animal in order to devour it. On the other hand, it was observed that the larvæ placed in moist earth absorbed the organic detritus, giving their intestine a distinct dark color. These facts led Lécaillon to assume that the larvæ of *Tabanus quatuornotatus* can feed, according to circumstances, on animal or vegetable matter in process of decomposition, and probably also on certain soft larvæ selected by the experimenter.

Lécaillon goes on to investigate the conditions of humidity favorable for the larvæ. Generally he left them in very moist earth. But once, having placed within the crystallizing dish, which contained the larvæ, another dish the under side of which was wet and rested on the former, from which the earth had been partly removed, Lécaillon found that fifteen larvæ had assembled under the smaller dish, between the two glass surfaces, and were completely submerged in water. He subsequently placed a number of larvæ in a cup filled with water, and observed that they appeared to be at home in the water, remaining in it and making no attempt to leave it. Later, Lécaillon placed the larvæ which he intended to rear in mud taken from an aquarium; they remained completely burrowed in this mud and could even find nourishment in it.

On the other hand, Lécaillon repeatedly allowed the earth in which larvæ were kept to dry out completely and found that the larvæ not only did not suffer but remained quite active.

His conclusion was that the larvæ of *Tabanus quatuornotatus* are capable of adapting themselves to a variable degree of humidity, and can live, at least for some time, in dry ground as well as in water. Concerning the range of humidity, and also concerning food habits, these larvæ, as probably other tabanid larvæ also, appear not to be adapted to limited conditions, in contrast to many other insects.

In his later publication, Lécaillon emphasizes the great resistance of the larvæ of *Tabanus quatuornotatus* to humidity and draught, explaining the fact by the presence on the body surface of a chitinous,

thick, and impermeable integument. In acetic sublimate, at a temperature of 24°, the larvæ remain alive for more than half an hour. It may be mentioned that many dipterous larvæ behave in this relation exactly like the larvæ of tabanids.

Lécaillon has again tried to rear larvæ of this species which had hatched on June 27, 1905. He found that they succeed best in moist ground. In February, 1906, a rather large number of these larvæ were still alive; they had grown comparatively little, measuring only 7 or 8 mm. in length, against 2 or 3 mm. at the time of their birth. Some lived for a year without having reached a much greater size than this. Some of the larvæ were still found alive in July, 1906.

Under the conditions mentioned, the intestinal tract of the larvæ almost always contains particles of soil, giving them a blackish color.

From these observations it is probable that in *Tabanus quatuornotatus* the duration of the larval stage is more than one year. However, Lécaillon believes that this need not necessarily be, as it is apparent that the larvæ when kept in moist earth do not find exactly the conditions under which they live in nature. Their growth is consequently much diminished and cannot be compared with normal growth. This subject, however, requires further investigation.

The chitinous cuticle of the larva was also studied by Lécaillon (1906). It is thick and resistant, as generally in dipterous larvæ. Its thickness in a larva of 630 μ in diameter being about 17 μ. This resistant and impermeable covering protects the larva efficiently. It may be left in the water, for instance, for several hours without being appreciably injured. It can be left, on the other hand, in dried soil for a considerable time without being killed. If one wishes to fix the larvæ *in toto*, even in the case of very small larvæ, great difficulties are encountered because of the impermeability of the chitinous membrane.

The free surface of the chitinous layer appears to the unaided eye to be smooth and devoid of hairs. But with a lens it is found that it presents numerous longitudinal parallel ridges and numerous hairs of reduced length. This characteristic has been found to be a general feature of tabanid larvæ, and the examination of the striæ can furnish the means to distinguish the genera and species.

The finer structure of the chitinous layer is easily studied on cross sections of the larva which is fixed and stained before. On section the chitinous layer is found to consist of three zones; first, an inner zone, which is in immediate contact with the hypothelium; second, a median zone, resting on the former; third, an external zone, which limits the body of the larva exteriorly. These three zones present different characteristics.

1. The inner zone is highly developed; it is much thicker than the two other layers taken together. It is of a lamellar structure, as on cross-section it presents numerous concentric striæ. This inner zone is slightly affected by acid stains.

2. The median zone is much less thick than the inner, but much thicker than the external zone. This zone or layer forms the thickenings which project exteriorly in the form of longitudinal ridges on the surface of the integument. It follows that it is much thinner at the level of the intercostal furrows than at the level of the ridges. It appears not to be lamellar in structure, but rather to present at the level of the ridges vertical striæ. Finally, its most remarkable property is its great affinity for basic stains.

3. The external zone is extremely thin; unless high magnifications are employed to observe it, it easily escapes detection. It covers the median zone in the intercostal furrows as well as on the ridges, and is not thicker at their level. It is not affected by either basic or acid stains. This layer forms the hairs. Neither the median nor the internal zone takes part in the formation of hairs.

It follows from the difference in affinity with regard to stains, shown by the two first layers, and the inability to stain the outer layers, that the three chitinous layers are easily distinguished by a combined staining method. By means of magenta red and picric indigo carmine, for instance, the inner zone becomes greenish and the median zone dark red, while the external layer remains colorless. Similarly, by a combination of hematoxylin and light green, the inner layer stains green and the median layer deep black, the external layer always remaining colorless.

The descriptions of the chitinous integuments of insects do not completely harmonize with those given by Lécaillon of *Tabanus quatuornotatus*. Lécaillon assumes two reasons for this fact: first,

the staining methods employed have undoubtedly been insufficient; second, the different types of insect may show important differences in the integument. If the integument is not very thick, it may often be of simpler structure. But only exact observations can determine this point. In the meantime, Lécaillon points out that the structure of the integument of this larva suggests strongly that described by Dubosq in the myriapods.

Lécaillon describes also the attachment of the muscles on the chitinous membrane of the larva of this species. The muscular fibers inserted in the integument do not end at the epithelium but cross the chitinous layer, becoming attached at the median zone. But the striation of their fibers disappeared where they reach the integument, and the whole section contained between the hypothelium and the median chitinous zone is formed by non-striated fibrils forming a sort of a tendon. All the muscles attached to the integument present this type of insertion. Lécaillon points out that the middle and external layers form the really hard and resistant part of the chitinous membrane of the integument, as Dubosq has shown in the myriapods. This aids in the understanding of the mode of insertion of the muscle fibers in the highly contractile larvæ of tabanids, in this region of greatest resistance. About Graber's organ, in this species, see page 35 and Plate 10, Figs. 111 to 116.

According to Bezzi, the *Tabanus* larva in which Paoli studied Graber's organ, may have belonged to this species (Paoli). See p. 37 (Special anatomy of tabanid larvæ).

Tabanus semisordidus Walker.—The egg masses of this South American species have been observed in British Guiana, by Bodkin and Cleare, in 1916, to be deposited on the leaves of aquatic grasses and in some instances on the leaves of young rice plants. They are laid in a little bundle consisting of twenty or more cigar-shaped, shining black eggs adhering to one another and to the leaf surface.

Tabanus solstitialis Schiner.—A common European species. Brauer (1880) states that the pupa is found in the water, being green in color and provided with large ear-shaped anterior spiracles. The larvæ are not mentioned.

Tabanus speciosus Ricardo.—In Madras, India, but rather rare; feeding on cattle with *Tabanus striatus* and *albimedius*, and similar in size and appearance to the latter.

According to Patton and Cragg, this species lays its eggs always on the leaves of water-lilies growing in deep water. The number of eggs laid is between 500 and 600. The process of oviposition is said to be similar to that of *Tabanus albimedius*, but *Tabanus speciosus*, instead of forming a V-shaped mass as is usually the case with the larger tabanids, lays its eggs in a round heap, which it then plasters over with a chalk-like substance, almost completely covering the eggs. The egg mass is figured (Plate 1, Fig. 19).

The larvæ of the larger Indian tabanids, including this species, are powerful swimmers and have air sacs connected with their tracheal tubes, so that they can float or sink at will. A description of the larva is not given.

Tabanus spodopterus Meigen.—A common European species. According to Schiner, very common in Austria, at the Neusiedler See. Brauer (1883) gives an illustration of the larva (Plate 6, Fig. 87, *a–f*), which he found, in the month of May, under dry leaves on the ground.

Tabanus striatus Fabricius.—The early stages of this species have become known through Mitzmain's work, which is the most thorough investigation into the life history of a tabanid species which we possess. We cannot enter here upon his results in as far as the habits of the adult fly and its part in the transmission of disease is concerned, but have to limit ourselves to report on the results obtained on the early stages of this species.

The eggs of *Tabanus striatus* (Plate 2, Figs. 22 to 27) were not found in the field, but Mitzmain was able to obtain them from females ovipositing in captivity. A large case was built for this purpose provided with a tank of water, growing plants, and two animals (carabaos) as a source of food for the flies. In a short time females were observed feeding on the hosts and several were found ovipositing in various places about the enclosure.

The time selected for egg laying under the conditions provided was invariably during the early afternoon, never later than 2 o'clock. This was observed in nearly fifty instances.

The eggs were laid in a compact mass either extended on a flat surface or surrounding various attached objects, usually of small diameter, such as projecting splinters of wood, suspended fibers of jute sacking, fine brass wire, a single animal hair, and coarse iron wire. Upon these materials the eggs were laid in an ellipsoidal form sometimes surrounding the objects completely or nearly so. On one occasion two egg masses were found upon a small splinter of wood which they entirely enveloped. The surfaces of the egg masses were continuous, so that the double mass resembled a single large one. When eggs were found deposited on a flat surface, on two occasions a leaf was the object selected. These were leaves of an ornamental plant which was used for shade purposes in the breeding cage. The plant grew close to the cement water tank in the breeding cage. In all other instances the eggs were deposited upon woodwork on the sides and ceiling of the cage, invariably upon the shaded portions, as the under side of beams and partitions. In egg laying upon flat surfaces there was a strikingly constant geometric form. Usually the form assumed was roughly a pentagon with a biconvex center.

At the beginning of oviposition (Plate 2, Fig. 24) usually two eggs are deposited in the position of an inverted V. Three to four eggs are then laid on either side of the apex of this V, and then one side and then the other is built up, rather irregularly at first, until the sides of the pentagon are completed. The eggs are laid cleanly and definitely, each line slightly overlapping the preceding one. When the eggs are laid in the extended order, they are deposited three or four layers in depth, but usually as many as six layers are required to complete the mass when the eggs surround a convex object.

In the process of laying, "the body is held away from the egg mass, the legs being planted firmly." When the eggs are attached to one of the objects mentioned above, the insect stands with head downwards, the fore legs suspended alongside the head, the hind and middle legs supporting the weight of the body. At the first movement, the anal end of the body is bent towards the thorax under the abdomen, and with a slight jerk the egg is laid, while the brush-like appendage of the ovipositor exudes a tiny drop of liquid coating the egg as it is deposited. The movement of deposition is very much like squeezing a bit of pasty material from a collapsible metal tube.

In several counts that were made by Mitzmain, the fly was observed to lay with clock-like precision at the rate of ten eggs per minute. This did not vary whether the attached object was above or below the fly. In three instances observed, the process occupied from forty to forty-five minutes. Both the beginning and completion of the egg-laying process were without deliberation, the insect walking away from the mass of eggs and flying off as soon as the last egg was deposited.

When disturbed during oviposition, the insect does not fly and can readily be carried without attempting to escape. While in the act of laying, if interrupted and dislodged from the position, it immediately begins to deposit a new egg mass. This was twice repeated with one female, and three distinct egg masses were deposited, all identical in geometrical arrangement.

The eggs of this species of *Tabanus* are laid with very little cementing material. The cement used is a transparent substance "and not dark and opaque as found to exist in the species described by Hine" (*Tabanus lasiophthalmus*). The cement provided by this species was tested and found to be water-proof, as well as insoluble in various grades of alcohol and xylene.

The eggs (Plate 2, Fig. 25) when laid are a pale clay-yellow, but within twenty-four hours become slightly darkened with an ashy gray tinge. Microscopically fine black striations can be seen running lengthwise for nearly 0.5 mm. from the end opposite the micropyle.

The shape of the individual egg is that of the muscid type with more sharply pointed ends; it is not quite spindle-shaped. Several eggs were measured and found to average in size 1.6 by 0.4 mm. The size of the mass varies from 9 to 12 mm. in length by 6 to 9 mm. in breadth.

The number of eggs laid in a mass varied greatly. In four masses counted there were respectively 270, 340, 417, and 425. Ten masses dissected from the bodies of killed flies were found to average 405; the greatest number found in any female was 495.

Mitzman gives a detailed description of the hatching process. Two egg masses were observed microscopically during the entire process of hatching, and fourteen egg masses were noted as to the length of the incubation period. The minimum period observed

was three days and the maximum five days. Four days is probably the average length of time required for incubation. It was observed that the degree of temperature and moisture influenced the time of hatching. Slight changes in either of these factors can be used to control the time of emergence from the egg. Mitzmain's observations on the hatching of an egg mass are the following:

"Twenty-two hours previous to the hatching of the embryo, certain unmistakable activities were discernible in the egg. The first signs of these were seen in the two eggs which formed the nucleus for the egg mass and which are the first eggs laid. These movements, as indicated by either of the dark eye spots, could be seen with a hand lens at intervals of a few seconds; their action was similar to that of the bubble in a spirit level. In about an hour the movement was seen to be rather general in the egg mass, accompanied in the eggs first laid by an alternate collapsing and distending of the exochorion. This action is the result of the torpedo-like movement of the head capsule of the embryo in the direction of the micropyle of the egg. The movement is effected by the piston-like action of the apophyses of the cephalopharynx, which appear to work alternately, bringing the saw-toothed mandibles in contact with the micropyle canal. These movements proceeded uninterruptedly during the hours of the night, the only change observable being that the body segments of the embryonic larva became better defined. At 4.25 the next morning the segments of the embryo could easily be counted through the chorion. The dorsal surface of the exochorion was seen to be slightly shrivelled."

"Fully one hour and thirty minutes intervened during which there was no action worth noting. This quiescence was interrupted by a sudden remarkable activity of all of the visible eggs of the mass. At 6.08 there was a general upheaval of the surface of the egg mass, an agitation within the eggs, and an alternate collapsing and distending of the eggshells. At 6.10 the first layer of eggs gave birth to a silvery horde of young larvæ, which at 6.12 had crawled from view [Plate 2, Fig. 27]. Then ensued another spasmodic agitation giving birth to another lot of larvæ, which crawled from the mass of empty eggshells [Plate 2, Fig. 26]. The emergence which is effected by the head structures is aided by the posterior protuberances, which functioning as prolegs push the body of the larva clear of the eggshells."

Mitzmain's discussion of the egg stage and of the hatching process is followed by a description of the morphology and habits of the young larvæ.

Immediately after emerging from the egg, the young larvæ (Plate 5, Fig. 80, *a*, *b*) seek concealment. In nature, no doubt, resort would be had to the convenient water course where aquatic plants, drift-

wood, and stones would be the probable hiding places. The larvæ under observation became very active and crawled out of the slender dish, a height of 9 cm., and fell into the water of the basin provided. When collected and placed in a deep glass vessel with some water, the entire mass took refuge behind the filter paper in the glass. There they crowded side by side with their syphons projecting from the upper edge of the paper. When disturbed and forced to take to the water, they were found in thirty minutes reassembled in the characteristic gregarious fashion behind the filter paper against the glass.

For convenience in study, a majority of the larvæ were transferred when 1 day old to individual glass jars one-third filled with clean wet sand from the lake shore and provided with strips of filter paper soaked in muck from the creek bottom. The jars, which were the common half pint jelly glasses recommended by Hine, were kept covered with filter paper, held in place by the tin lid which had a disk cut from its top to admit air. By renewing the moisture on the strip of filter paper in the jars, the filter paper cover serves ideally to control the humidity.

A considerable number of the larvæ were not separated, but were left together for observation in a glass dish with a few strips of paper saturated with muck from the creek. The young larva is briefly described as follows:

"The larva one hour after hatching [Plate 5, Fig. 80, *a*, *b*] is 1.5 mm. in length. The following day several were found to measure 1.8 mm. The general color is a dirty white with a tracheal system of waxy white, the abdominal contents pale green, and the Malpighian tubules of a lilac color. There are 2 black eye-spots located midway on the head capsule. The latter tapers to a sharp-pointed mouth with a prominent pair of great hooks or mandibles. The segments are provided with typical, conical, truncated, prolegs, each armed with a chaplet of medium long, brown hairs. The siphon which is carrot-shaped at this stage is a prominent feature."

Food in a variety of forms was furnished the larvæ. They thrived from the start on minute crustaceans, larvæ of *Stomoxys*, mosquito larvæ, and young angleworms. Full grown angleworms were found unsuitable, and larvæ of the blow-fly and flesh-fly were not satisfactory unless killed previously, as they were capable of killing or in-

juring even well developed *Tabanus* larvæ. As soon as the insect becomes aware of the presence of food, the claw-like mandibles are protruded from the head capsule, and bury themselves in the live food like meat hooks. With a slight curve dorsally, the larva's body is brought forward, and a small portion of the food is lacerated. This is aided by a twisting of the head and a pulling with the extended jaws. The mandibles are brought together with a rapid clawing action, the parts working in apposition. When prehension is effected, the jaws move alternately upwards and downwards and laterally, and the bolus is swallowed in fibrous strands.

Seeking and devouring food is not a continuous operation as it is in the case with *Stomoxys* and the dung-flies. The *Tabanus* larva requires a long rest after a sufficient meal is taken. A 2 day old *Tabanus* is capable of devouring two half grown larvæ of *Stomoxys* in twenty-five minutes. In one instance a full grown *Stomoxys* larva was destroyed in exactly twenty minutes. Here the attack on the *Stomoxys* was made through accidental collision, the *Tabanus* instinctively thrusting out its mouth and tentatively taking a bite. It apparently became greatly excited (this was its first meal), and thrusting its head into the body of the *Stomoxys* larva, commenced to probe by twisting its head rapidly. In less than a minute the cuticle was broken through and an ample slit was made through which the entire head was buried in the body of the victim, whereupon an energetic gouging took place. The *Tabanus* worked through the cephalic third of the body upwards to the head, then worked in the other direction on the lower two-thirds. This gouging was continued until the *Stomoxys* had become completely eviscerated, during which time the head of the *Tabanus* kept steadily probing, twisting its pharynx from side to side, and pushing forward with its rostrum until the *Stomoxys* larva was completely devoured with the exception of the cuticle.

The full grown *Tabanus* larva does not wait for its food, as is the tendency in the young stage, but actively pursues its prey. When an angleworm is seen, perhaps 2 mm. distant, the elastic head capsule of the larva darts forth, curves its claw-like hooks about the worm's body, and with its head curled under its struggling prey, retreats quickly into the sand until all but its cephalic end is hidden.

It begins to feed then, devouring in twenty minutes an angleworm fully four times its own length.

The intestinal tract seen through the hyaline cuticle soon partakes of the color of the food ingested. The color is pale brown when the food consists of the wet muck in which crustaceans and minute forms are sought. As a result of feeding on blow-fly larvæ and angleworms, the young *Tabanus* assumes a variegated color. The intestinal tract then appears tinted with green, yellow, brown, and red particles of the food.

In one set of larvæ, as Mitzmain says, "The origin of cannibalism as an acquired habit was observed." This was seen in larvæ which had been kept together for four days since their birth. Until that day no food was offered them except that which they might have obtained from the surrounding creek water. Apparently they lived together amicably with their bodies compressed against the glass dish and the bit of filter paper. A live angleworm was placed in the glass dish while the resting larvæ were observed with a lens. The worm was not placed in the immediate vicinity of the mass of larvæ, but nearly 4 cm. distant. The presence of the food appeared to act as a stimulus. No movement was made towards the worm, but each larva appeared to become greatly excited and began to prod the larva nearest to it and to nip its neighbor's appendages; several instances of laceration were noted. This doubtlessly marked the beginning of systematic cannibalism. From this cause, 39 of the 365 larvæ kept in the large glass dish were destroyed within four days. Four dead bodies were recovered. Upon another occasion the extent of cannibalism was much more marked. A lot of 415 larvæ which hatched on November 12, 1912, was placed in a deep glass dish with moist lake-beach sand, and fed daily on angleworms. Each morning it was observed that only about one-half of the worms supplied the previous day were eaten, so that with the daily fresh supply more than enough food was present. Another lot of 300 larvæ, the same age as the preceding, was kept in individual glasses under similar conditions. On December 6, counts were made of the survivors in the large glass dish. Thirty-five larvæ remained, of which eighteen were the maximum size, eleven were a little more than one-half this size but equal to the largest found in the individual

jars, and the remaining six larvæ were so small as to be easily overlooked. The count of the larvæ from the individual jars showed a loss of twelve, or less than 5 per cent. Allowing 5 per cent for loss from other causes, it appeared that above 85 per cent of the larvæ kept together in the large jar was destroyed through cannibalism.

It has been observed by Hine in other species that a *Tabanus* larva is enabled to survive for a few days in the absence of food. In this species, likewise, there seems to be a decided resistance to starvation, two instances showing periods of ten and twelve days.

Mitzmain found the movements of the body to be in general similar to those of larvæ of the muscid type. There is a general progressive peristaltic movement, invariably accompanied by a decided telescoping of the segments. The head is raised as the prolegs of the anal end push the body forward, then it is lowered. The mouth is projected when the head capsule is extended, but recedes quickly when the glass sides of the container or any obstacle is encountered. The larva can easily move backwards for a considerable distance. This it does if wedged in a tight place or in capturing food when it retreats into a channel previously made in the sand.

The larvæ readily adapt themselves to a watery medium. They can remain submerged for several minutes at a time without apparent discomfort. When placed in deep water the movements of the body consist of a general struggling without apparent definite purpose. At any rate, there is little or no progression, the body doubles like a bow, the head and tail meeting, then straightens with a whipping action. In swimming, the body is held along the surface of the water and the syphon is extended towards the air in a manner suggestive of the larva of an anopheline mosquito. The principal movement observed is that of simple telescoping of one segment into another. When speed is required or an obstruction is to be passed, there is a vigorous whipping movement of the syphon laterally, towards and away from the head. This latter movement is also noted when the insect is disturbed.

When a young larva is placed in water containing entomostracans or other minute animals, a barely perceptible churning of the liquid occurs in the region of the mouth. This disturbance is no doubt caused by the movements of minute tentacles which assist in procur-

ing food. These tentacles form the armature of the stomal disk, consisting of a process arranged like a turnstile mounted on a pitted chitinous plate at the base of the great hook or mandible. In the very young larva the stomal disk appears as a chaplet of delicate chitinous rods. When a larva is treated with strong caustic potash, the stomal disk appears to be the only structure which resists its action, the other chitinous structures, even the heavy pharyngeal apophyses, are bleached. In common with the other chitinous portions of the head capsule the stomal disk is shed at each of the three ecdyses.

An account of the general development of the larva follows:

The young larva shows in its form and behavior its adaptability to an aquatic life. This is well illustrated when a larva is placed in an aquarium containing mosquito wrigglers. The *Tabanus* has no difficulty in keeping afloat with them and foraging at will upon the active culicid larvæ. *Tabanus* larvæ have been observed capturing wrigglers, holding them by their jaws under the water, and actually killing the culicid through drowning. In one instance a *Tabanus* larva held its victim, which was fully five times its size, suspended beneath it in such manner that the culicid was unable to project its syphon for breathing purposes, while that of the *Tabanus* was functional. The *Tabanus*, obtaining a secure perch by dragging itself and the prey above the water, devoured the mosquito wriggler in a few minutes. In another instance the weight of the culicid pulled its captor under the water to the sandy bottom a distance of nearly 30 cm. Here the *Tabanus* showed its superior vitality by remaining attached for nearly two minutes until apparently assured of the immobility of its prey, then, releasing its hold, the *Tabanus* larva struggled to the surface where it rested with syphon extended. The mosquito larva meanwhile moved feebly several times, and succumbed within a few minutes.

This adaptability is lost, however, in the developed larva which becomes more slothful in movement and grub-like in superficial appearance. Both extremities, the head and the syphon, become obtuse in form, and the ventral protuberances functioning as prolegs become more truncated. Growth after the second molt becomes noticeably less in length and more in thickness. The greatest growth

observable was shown to be between the periods of the first and the second molts.

The following table is given by Mitzmain to show the normal growth of a larva. The measurements and the critical stages of life are indicated.

TABLE I.

Progress of Development of a Larva.

Date.	Length.	Stage of development.
	mm.	
Sept. 15	1.5	At birth.
" 16	1.8	1 day old.
" 20	3.0	
" 21	4.0	
" 22	5.0	
" 23	6.5	
" 26	11.0	After first molt.
" 30	20.0	
Oct. 8	22.0	
" 9	25.0	Second molt.
" 12	27.5	
" 17	29.5	Mature larva.
" 21	27.5	
" 24	17.5	Pupa, third molt.

In all biological accounts of the Tabanidæ there appears to be one phenomenon which is uniformly noted. This is the remarkable difference in growth shown by individual flies of the same species. The only process in the development which seems to be synchronous is the hatching of the eggs. After that the variations in time of development are extreme. In *Tabanus striatus*, for example, some larvæ twelve days old measured 3 mm., while others under precisely the same conditions measured fully 11 mm. In another instance two flies emerged as well developed imagos October 31, while twenty-seven of the same brood still remained apparently healthy in the larval stage December 20.

Mitzmain is the first to mention molting or ecdysis in tabanids, stating that he has been unable to find, "in the very meager literature available," any reference to the molting process in Tabanidæ. It is referred to indirectly by King at Khartoum, who found in

Tabanus biguttatus the shed larval skin adhering to the "puparium."[26] Mitzmain had, however, not seen Paoli's work on the organ of Graber, in which also mention is made of moltings.

By Mitzmain the process of shedding the skin was observed in *Tabanus striatus* in a great many instances. The time of molting of a brood of larvæ is extremely variable, which is consistent with the great variations noted in the time of development in general. The process has been accurately noted in two individual larvæ, although observed superficially in numerous others. The three molts are similar in their general aspects, the main distinction being the more profound changes produced in the insect at the later molts.

The usual preparations for molting were observed in this species. The premonitory signs were the refusal of food, uneasiness when exposed to light, desire to find a remote corner, and finally the stiffening of the cuticle. In one instance the larva was found in one spot pressed against the glass for three days. Here, between the sand and the glass of the jar, an excrementous cement was used to fasten the end of the abdomen. This material holds the end of the body very securely, although the remainder of the body requires free lateral movement. By the time the ecdysis is completed, the head has moved 3 mm. from the spot where preparations for the process were made, while the anal end has retained its original position.

The shedding of the skin usually requires several hours; in one instance, due no doubt to interference by the observer the time was nearly twenty-four hours. In the first and second molts, splitting of the cuticle begins at the thorax, resulting in the tearing out of the entire head capsule which adheres to the molt during the remainder of the process. The anal segments are molted finally and the larva, emerging in its new skin, crawls its length on the cast skin and rests alongside it for two or more hours.

The first molt begins with larvæ 7 days old, the majority molting before the tenth day. The second molt usually occurs after an interval of at least four days, and in some larvæ as late as eight days,

[26] The use of the term "*puparium*" for the tabanid pupa should not be encouraged. The tabanid pupa is a true pupa, in contrast with that of muscids, or syrphids, where the true pupa is enclosed in the last larval skin, justifying the name puparium to differentiate it from a free pupa.

when fifteen to eighteen days old. The time of the third molt precedes immediately the appearance of the puparium. This period, as has been noted, shows the greatest diversity among individuals of the brood. The third molt in twelve instances was shed between the ninth and twelfth days of life.

In other individuals the process was not completed within three months, yet the adult fly was an apparently healthy insect.

Certain unimportant changes in morphology, dependent on the molting process, are noticeable. The loss in size due to contraction of the cuticle preparatory to ecdysis is usually compensated by a substantial extension immediately following the process. The extent of shriveling of the cuticle is represented by 1 mm. in the first molting, 1.5 to 2 mm. in the second stage, and 2 to 3 mm. preparatory to the third stage. There is a notable increase in length resulting from the second ecdysis. A larva, measuring 22 mm. on the day previous to the shedding of the skin, measured fully 25 mm. the following day. In measurements of this sort one must make allowance for the extraordinary amount of telescoping of segments. As much as 5 mm. may be involved in this process.

The structures mainly involved in the ecdysis are the tracheal system and the appendages of the head. The anal ring of the trachea constituting the syphon is drawn off in each molt in a perfectly cylindric form. The body trachea is torn from its connections in irregular strands. The entire head capsule, including the chitinous pharyngeal framework, the great hook, and other mouth structures, are found in perfect form in the various exuviæ. These parts upon renewal in the larva become more heavily reinforced.

The exuvia (Plate 5, Fig. 81, *a*, *b*, *c*) is usually in a good state of preservation; crumpled, to be sure, but it can be extended in alcohol to three-fourths the length of the larva. Following each ecdysis, the larva is invariably leaden gray with tracheal strands of waxy white. Three anal segments including the syphon become lead-colored and stiffened in structure. They are at this stage more truncated, with an anal band of cuticle 1 mm. in depth, making the syphon appear somewhat atrophied. This is no doubt consistent with its restricted function. The color of the viscera has changed from the brilliant red and yellow to an indeterminate white, and the

lilac tint of the Malpighian tubules has changed to a salmon color. The latter changes are due probably to a clearing process, in which the larva indulges during the quiescent stage preceding each ecdysis.

After the second molt the fleshy protuberances functioning as prolegs become reinforced with a slight cuticular ring at their bases. The mouth-parts at this stage are heavily chitinized. The great hooks or mandibles show a marked serration of the biting edge. The head projects more, exposing the dark brown ocelli, which, prior to the second molt are seen only through the cuticle of the thorax situated nearly on the middle of the concealed head capsule.

The signs characteristic of the final molt which results in pupation are refusal of food, restlessness, attempted migration, and finally burial in the sand at the bottom of the jar. The body decreases slightly in length, but the thickness remains the same.

On the extremity of the abdomen, tiny tubercles appear which project more from time to time, becoming tapering and spike-like. Near the caudal end of the abdominal segments, roots of hairs appear. These at first resemble brown spots of pigment and gradually lengthen into stiff brown hairs. The cuticle on the body becomes stiffened and shingle-like at the joints of the segments. The latter telescope less, and one can see numerous particles of sand embedded in the joints of the segments. These sand particles have been carried in during the telescopic movements of the abdomen.

After the fully developed larva passes through a period of semi-dormancy buried in the sand, the skin is seen to be ridged with cuticular plates. The head region is reinforced by stiffened cuticle, and the mouth orifice is closed by a plug of hard rose-colored cuticle. This pigmented material lines the entire pharyngeal sinus, plugging the mouth and the cephalopharynx. The cuticular plug has a substantial fold which forms a slit for the passage of the molting mouth. Caudally a similar impervious mass closes the opening of the syphon. A cuticular collar strengthens the base, and the connective tissue surrounding the trachea of the tract of the syphon tends to contract. Then the supports of the central trachea are gradually cast loose by a gentle wriggling of the insect's body. About this time there is a general wrinkling of the epidermis, the folds telescoping upon each other, and the surface becomes like parchment.

Synchronous with the primary contraction of the segments, a light pea-green suffuses the last three segments of the body. The remainder of the larva changes to this color over night. By morning the abdominal segments have changed from green to ocher, when the molting of the cuticle ensues. The shedding takes place in sections. The chitinous framework of the head is thrown off like a hood. This portion is everted upon the body, and remains dangling from the exuvia during the process. One-half the length of the skin is loosened on the side opposite that to which the chitinous framework of the head is attached. This is shed by a peculiar auger-like movement of the tail end which is not attached to the glass or other object in the container, as in the previous molts. The skin is virtually unrolled from the detached head to the anal end, where it lies in a crumpled heap. Then the skin of the other side of the body begins to be shed. The chitinous framework constituting the former head capsule of the larva becomes rolled up in the exuvia, while the skin is torn slowly from the new membrane. When the first half of the skin is peeled off to the anal tip, the cast skin becomes attached to some object. In this instance the glass of the jar served as an anchorage during the remainder of the ecdysis.

The upper half of the body of the newly molted larva is encased as in an armor in pouches and pads of integument, outlining in a gauzy film the future appendages of the fly.

Mitzmain's description of the full grown larva (Plate 5, Fig. 80, *c*) is given below:

"The length is 28 to 29.5 mm.; the width, 3 to 4 mm. The anterior half of the body is a greenish yellow, the remainder is a dirty white. At this stage the form is grub-like."

"The head capsule, which occupies one-fifth the length of the larva, is a cylindrical bulb, formed by the invagination of the thoracic ectoderm. It supports the eyes, the antennæ, and the mouth parts. It is bound by a framework of chitinous rods, the cephalopharyngeal apophyses. This structure, observed through the thorax when the insect is in action, is composed of four black, medium-thick, skeletal pieces running the length of the three cephalic segments in the form of a pyramid, with its apex provided with the external mouth parts. It terminates in the claw-like mandibles which are similar in color and texture."

"The mandibles [Plate 5, Fig. 79] are heavy, powerful structures, slightly serrated on their inner surfaces. The musculature of these appendages permits the

two elements working in apposition. At rest they are held horizontally, and can be projected suddenly and thrust vertically downward, which is obviously of great assistance in grasping the prey."

"The palpi and antennæ in this species are silvery white, and usually found glistening with moisture."

"The eyes are oval in shape, with the long axes parallel. When the larva is prepared to molt, the pigmented spots are usually distorted. In this species the eye spots or ocelli are very prominent, especially in the younger stages of the larva. They can be first seen in the embryo where they appear as dark beaded structures through the chorion of the well developed egg. In the young stage of the larva the eyes appear in the pharyngeal cavity midway between the mouth and the cephalopharynx, and as growth continues they are located nearer the distal end of the head capsule; so that when the larva is full grown and the mouth structures protrude in locomotion or prehension the eye spots are seen to project on the head capsule with the mouth parts."

"The trachea which terminates in the conical tubular syphon is lead gray in contrast to the dense white of that portion anterior to the anal segment. Anterior to the syphon there is a cuticular cellar of slightly darker shade."

"The prolegs are formed by truncated projections, six in number, three on each side of the midventral line and extending laterally. Each protuberance is provided with a tuft of short, fine, brown hairs. These hairs appear to be surrounded by a secretory substance, which is slimy in character."

"At the base of the syphon, beneath the cuticle on the dorsal side opposite the anal capsule, is a tiny structure which attracts attention on account of its movements and peculiar arrangement. In the newly hatched larva it is a process composed of four lustrous black disks arranged in two pairs, one in front of the other, and set in a mass of fat bodies. The larger of the disks, the anterior pair, is less than 0.1 mm. in diameter. The movement of the process is similar to that of a pendulum, and is active only when the larva moves. With each molt these disks become smaller and increase in number. In the full grown larva the process becomes a triangular mass of loosely arranged beaded disks. They appear to be mere specks of pigment beneath the skin, but their structure and action are so constant that either the process is characteristic of the species or investigators have overlooked or ignored them in other species."

The description of the pupa (Plate 12, Fig. 152 *a*, *b*; Fig. 143, *a*, *b*) after Mitzmain is given below:

"The average length is 18 mm., and width, 3.5 mm. The color is pale brown, the last 2 segments of the abdomen being slightly darker. The head tubercles are not clearly defined; color, dark brown. The prothoracic spiracular tubercle is slightly elevated, oblique; rima, salmon-colored and crescentic in form."

"The first abdominal spiracle is perfectly round and larger than the others, which are slightly ovoid; the rima of all the spiracles curves from above posteriorly."

"The terminal abdominal segment [Plate 12, Fig. 143, *a*, *b*] shows a sexual distinction in the arrangement of the short spines midway on the ventral side, anterior to the terminal teeth. In the male ten to twelve of these spines form a continuous serrated border. In the female the spines occur in two groups of four to six spines similar to those of the male, but separated by space equal in width to that of one of the groups."

"The terminal teeth of the posterior segment are arranged with two pairs close together on the dorsal side and one pair on the ventral side. These teeth are black-tipped and acute; all of them are directed slightly outward. The lateral teeth of the two dorsal pairs are the longest. The ventral pair is smaller and is set slightly in from the periphery of the segment."

"After the final ecdysis which results in the formation of the puparium, the nymph, at first a light green, gradually changes to yellow. Upon the second day, the eye spots change from yellowish to pale brown, then to a chocolate color. Beginning with the third day the pads of the wings and the legs, at first light brown, assume the same color as the eyes. The chitinous pad enveloping the wing is densely opaque, so that only the plications of the developing wing can be discerned. Upon the penultimate day, the fifth or sixth usually, the abdomen, which heretofore has been a uniform yellow-brown, becomes striped with light orange and brown, which colors gradually deepen until the time of emergence."

The process of emergence from the puparium is also described by Mitzmain (Plate 12, Fig. 152, *b*):

"In emergence, the puparium which lies buried to some depth in the sand is invariably dragged to the surface where the final acts of emergence are completed. Two or three days prior to the act of emergence, the puparium shows considerable mobility when disturbed by handling or stimulated by light. Certain movements, which one learns through numerous observations to be characteristic, can be considered as actually premonitory. These occur usually from ten to twenty minutes prior to the breaking of the cuticle, and serve the observer as warning signs. If during this interval a low power lens is focussed on the compound eye, the epidermis of the fly separating from its connective tissue fastening of the puparium can easily be seen. This action resembles strikingly a wave of water moving between the walls of the puparium and the epidermis of the fly. It may be considered as the movements of a semiliquid layer between the fly and its puparium. Another movement, which can be observed within a few minutes after that previously described, is the momentary contraction and expansion of the sides of the abdomen between the two lateral ridges. This too, no doubt, is effective in tearing the connective tissue lining to facilitate emergence. A few minutes later the anal end of the abdomen is torn loose from its fastening, and emergence of the fly begins."

Since the puparium is unrolled from the head, the compound eyes are soon exposed to view, so that the sex of the fly may be distinguished. The appendages, antennæ, palpi, and mouth-parts are dimly visible. The head appendages are freed primarily by the spasmodic wriggling of the abdomen, but the labellum, which is seen to become turgid and flaccid in turn by the injection of air into, and withdrawal of air from, the "extensive tracheal sacs which lie in the cavities," and the erectile stomal disk through the pressure downwards against the walls of the puparium, assist also in the process. That these head appendages assist effectually in the emergence is evident from the lines of cleavage in the enveloping membranes.

The puparium splits on the median line of the thorax; simultaneously the hood enveloping the head drops by a sternal hinge. The labellum can be seen still pressing upon the interior of the hood as the head emerges. In a minute the wings are rent from their envelopes by the sturdy pressure of the legs, which have slid out of their sheaths simultaneously with the cleavage of the thorax. The legs directly assume their normal position, and the fly walks forth bodily, spreading its plicated wings. The liberated wings show a clear expanse of unwrinkled membrane which at first is soft in texture and clear lead-colored throughout. Finally the inflated abdomen appears in the dorsal slit, and at once is drawn clear of the encumbering puparium.

The time from the appearance of the head to the evacuation of the puparium requires less than two minutes. This time is increased a minute or two whenever the wing sticks to the lining of the puparium, resulting usually in a torn wing.

Directly after emergence the wings are shorter than the body, but, constantly vibrating, they gradually lengthen, whereupon they become hardened and prepared for flight. The fly does not spend any time preening itself, as is the case with some of the Muscidæ at this stage. The time prior to flight is spent, however, in a clearing process. This begins with a copious discharge of meconium within three to five minutes after emerging. At first the defecation is performed at least five times per minute, then once per minute for a period of twelve minutes. At the end of this time the excretions become more watery in character. In the meantime the fly walks

about in a restless manner, constantly vibrating the balancers and flapping its wings, while the distended abdomen becomes reduced to more normal proportions.

The meconium, which is deposited in large quantities, is pale brown in color, rapidly changing to amber, then becoming clear. The primary, heavier excretion appears decidedly oily in nature, when examined with the microscope.

In from fourteen to twenty minutes, voluntary flight takes place. This is at first tentative, the insect alighting upon the floor about a meter distant. After a minute of rest, flight is resumed, the fly escaping through the open window.

The puparium left behind shows certain points of cleavage which prove to be very constant. There is a dorsal slit on the median line of the thorax which extends nearly the length of the notum. Another slit extends midway across the orbital region through the genæ to the wing pouches. A third slit extends between the two wing envelopes, and a slight one behind the prothoracic spiracular tubercle.

In the thirty-two emergences recorded, the males preceded the females by an average of half a day. The males spent from three to seven days in the pupal stage, averaging five and one-half days, while this period required four to nine days with an average of six days in the female flies.

Bainbridge and Fletcher (1914) observed *Tabanus striatus* near Madras, and give the following statements which may serve to complement Mitzmain's work in as far as it seems based on field observation:

> "Eggs are laid in a large mass, usually on a leaf or twig overhanging water into which the young larvæ drop on emergence, thence-forward leading an aquatic life burrowing in the mud at the water's edge and feeding on worms or living or dead insects. The full grown larva is 40 to 50 mm. long, dull whitish, elongate, tapering at each end with protuberances at the edges of the segments. When full fed it leaves the damp mud at the water's edge and after a quiescent period pupates in the earth above water-level."

An egg mass of *Tabanus striatus* on a paddle leaf is figured (Plate 2, Figs. 22 and 23).

Patton and Cragg state that *Tabanus striatus* oviposits as a general rule on blades of grass, pieces of stick, etc., at the edge of a river, stream, or pond.

The larvæ are said to be powerful swimmers having air sacs connected with their tracheal tubes, so that they can float or sink at will.

Large numbers of egg masses are regularly destroyed in Madras by a small chalcid parasite which was not identified.

The eggs are brownish white when deposited (Patton and Cragg).

Tabanus stygius Say.—A species of the Middle and Southern States, recorded from Massachusetts, New Jersey, Illinois, Ohio, etc., the life history of which is comparatively well known through the observations of Hart (1895) and Hine (1906).

On its oviposition and egg Hine is the first to give us information. The species oviposits principally on the leaves of *Sagittaria* standing in shallow water, habitually placing the eggs just above the point where the petiole meets the expanded part of the leaf (Plate 1, Figs. 3 and 4).[27] The precision with which this habit is followed becomes a matter of interest. Out of hundreds of masses of eggs observed, only a few were placed on other species of plants or in a different position on the leaf (Plate 1, Fig. 5). The female is occupied for a half hour or more in placing the several hundred eggs composing a single mass, and during this time the observer can take a position close by and watch the proceedings without frightening her away, but species of *Tabanus* are more particular about the approach of intruders than are various *Chrysops*.

The egg mass is white when first placed but soon turns brown; it is convex, and is composed of about five layers, one above the other. Individual eggs are nearly the same size as those of *Tabanus atratus*, and are similar to them in form. Hatching as observed, occurred in seven days after oviposition. From a careful study of microscopic sections of eggs killed as soon as laid, it was concluded that development does not begin until after oviposition, consequently the time given is the entire incubation period.

When first hatched the larvæ contain a considerable amount of unused yolk, which furnishes them food for a time; it is therefore unnecessary for them to eat anything for a few days. This is ad-

[27] The leaf represented in Fig. 4, is a leaf of *Peltandra*, similar in shape to the *Sagittaria* leaf (Plate 1, Fig. 3).

vantageous no doubt, for food is not always at hand, and in case it is not, the fact that nourishment is furnished naturally gives them an opportunity to investigate their surroundings.

At hatching time nearly all the larvæ that come from a single mass of eggs appear at the same time and when they have freed themselves from the shells, go tumbling down into the water, scattering more or less and sinking to the bottom, where it is difficult to observe their further actions.

Hine found that small catfishes (*Ameiurus melas*) are enemies of these larvæ, and he observed two of them devour 200 young larvæ of *Tabanus* within a few hours.

Hine's attempt to rear the larvæ from the egg to the adult was not successful. On July 21, a number of larvæ just hatched were placed in a breeding jar containing damp sand covered over the top with fine plant material, and small crustaceans were put in for food. The larvæ took kindly to the surroundings, accepted the food offered, and began to grow at once. After about two weeks, as angleworms were much easier to obtain, these were substituted for the crustaceans, with no bad effect on the larvæ, which continued to grow, though rather slowly. The largest attained a length of about 10 mm. by the beginning of winter, when they ceased eating. They appeared to be in good condition in the spring, but for some reason died without further increase in size.

On August 2, of the same year, Hine took a large larva of this species in Summit County, Ohio, from under a flat stone along a brook that ran from a spring. When taken this specimen measured over 40 mm. in length and had every appearance of being mature, but it continued to eat the angleworms given it until late in the fall. It then ceased feeding until the following spring, when it took a small amount of food and entered the pupal stage about the middle of May, the adult, a male, issuing June 14.

Hine thinks that it is hardly possible that this species passes all its transformations in a single year, for the larvæ reared from eggs were not over 8 mm. long when the specimen over 40 mm. long was collected; and as the latter did not produce the adult until about the normal time for adults to appear under natural conditions, it does not seem possible that the first mentioned larvæ could have reached maturity and produced adults before the second year.

The young larva is briefly described by Hines as follows:

"Larva, when first hatched, 4 mm. long; entirely light colored; form as in older specimens. As growth continues size is the only noticeable change."

The mature larva has been figured and described in detail by Hart (Plate 3, Fig. 40). This species was the most abundant tabanid larva found in the vicinity of Havana, Illinois, in the spring of 1895. It first appeared in Hart's collections September 14, 1894, when a number were noted swimming among vegetation near the margin at Station B, on the wet springy shore of Quiver Lake, with sandy and muddy ground, grass, and coating of algæ. In the spring they were, however, found at nearly all of the stations, but more particularly in connection with tipulid, muscid, and *Eristalis* larvæ in matted accumulations of dead stems and leaves over mud. They were especially abundant on March 30 in Flag Lake, where large plump larvæ appeared at every turn. It was a surprise to find a few of them upon the bottom in the open shallow water, far from shore, in the middle of Quiver Lake at Station A, for which locality Hart gives the characteristics: "Shallow, mud and sand, grass and floating vegetation, variable." Young larvæ have been common in connection with larvæ of *Bittacomorpha* and *Limnophila*, at Station I (see *Tabanus atratus*) since March 17. At others of Hart's stations they have been common in moist drifts of fine rubbish washed up by waves. Pupæ were formed in the breeding cages May 10 and 23. One emerged May 27, and another tried to emerge June 2, but died and was removed from its case.

The larvæ of *Tabanus stygius* resemble, according to Hart, those of the *lineola* group in their striation and coloration, but differ in their short lateral prothoracic areas and larger size. They are like *atratus* in size, but may be readily separated from it by their coarser lateral striation, straw-yellow tint, slender lateral pigmented stripes, and usually projecting terminal stigmatal spine.

Hart's description of the mature larva follows:

"*Larva.*—[Plate 3, Fig. 40.] Length 45–55 mm., diameter 6–7 mm. Bright straw-yellow, varying in some young larvæ to nearly clear white; marked with light fuscous brown microscopic pubescence, usually paler at each stage than *atratus*."

"Lateral prothoracic striated areas are not more than half as long as the dorsal, striation not finer than that of the middle and lower lateral areas of the mesothorax, striated portion shining; a small smooth spot adjoining the impressed line below; remaining upper lateral thoracic areas a little less coarsely striated, but not strongly different from that of the prothorax; abdominal lateral areas a little more finely striate; dorsal and ventral areas with margins striated, disks nearly smooth in adult larvæ, last segment more strongly striate, especially beneath."

"Dark annuli distinct, broad, including false feet, a distinct transverse dorsal and ventral pale spot in front of the false feet; abdominal annuli often with a small triangular backward prolongation on median line above. Prothoracic lateral space occupied by a pale brownish fuscous quadrate spot in front of the striated space. Meso- and metathoracic lateral stripes usually distinct, but slender, scarcely dilated posteriorly, lateral edges of dorsal areas diverging; lateral stripes of abdomen almost wanting, except on last two or three segments. In these stripes the punctures of the upper and lower rows are indicated by rounded pale dots, and those of the inner rows by elongate dots. Last segment with bases of respiratory tube and anal prominence encircled with dark rings; joined by a lateral connection, its dorsum with at most a short basal line or pair of dots on each side. Coarser pubescence of false feet tipped with pale brownish."

"Main internal tracheæ thick and noticeable, especially in young larvæ, lustrous, subparallel, not strongly sinuate, nearly straight posteriorly; terminal stigmatal spine dark reddish brown, smooth, usually protruded."

The pupa (Plate 11, Fig. 125; Plate 13, Figs. 153 and 161) has been described by Hart as well as by Hine, both of whom reared the adult from pupæ obtained from adult larvæ taken in nature. The following is Hart's description.

"*Pupa, female.*—Length about 30 mm., diameter about 6 mm. Light brownish fuscous, thorax paler, shining, abdomen roughly transversely wrinkled, and subopaque. Palpal sheaths distinct, as far apart as are the setæ borne by the larger tubercles at the center of the anterior surface of the head [Plate 13, Fig. 153]; surface between them rounded, bearing a small wrinkled tubercle at middle; antennæ and tubercles darker than surrounding surface; ocellar tubercles distinct; prothoracic spiracular tubercles slightly but evenly elevated in a plane parallel to that of the surrounding surface; rima nearly straight in its outer half, inwardly curving strongly forward, and ending in a conspicuous hook; free margin of tubercle rounded at tip."

"First abdominal with two distinct setæ each side above the spiracles; abdominal spiracular tubercles rounded, broad behind, low-subhemispherical, rima long, following posterior border of tubercle, slightly curved at middle, more strongly curved forwards at each end; on anterior surface a transverse groove extending across the tubercle, but not as long as the rima. Fringes of unequal

spines, often tipped with blackish, all but two of the long spines wanting in a broad space above on seventh segment. Terminal teeth [Plate 13, Fig. 161] nearly equal, tipped with blackish, their points marking the angles of a hexagon, slightly wider than high. Ventral fringe of last segment not webbed together; lateral tufts high—on a level with upper lateral line."

Hine's description is shorter:

"*Pupa* [Plate 11, Fig. 125] 29 mm. long; color dark, approaching fuscous; prothoracic spiracle strongly bent at the middle; rima oblique and straight for the outer half of its length, remainder gradually curved, with a broad hook at the inner end. Teeth at the end of the abdomen [Plate 13, Fig. 161] six in number, nearly equidistant from one another, of nearly the same size, with the extreme tips slightly turned inward."

The pupa of *stygius* is, according to Hine, much like that of *sulcifrons*, but there is some difference in the prothoracic spiracles and in the abdominal teeth.

Tabanus sulcifrons Macquart.—A North American species inhabiting Pennsylvania, New Jersey, Ohio, Illinois, and Louisiana. Closely related to *Tabanus exul* and *abdominalis*, and like the two latter, appearing late in the season, in Ohio abundant in the latter part of July and all of August, in Louisiana recorded from September 7 to October 16.

Hine has made many interesting observations concerning the habits of the adult flies of this species, but was unable to work out its life history. Although the eggs were procured in many stages of development by dissecting the females, the habits of oviposition have not been observed. The form of the eggs and the number produced by a single female are the same as in other species of its size. Specimens containing eggs almost fully developed were taken in various places, but Hine could not get any clue as to where oviposition occurred by dissecting the females where they were collected, as he had hoped to do.

The pupal case of the species was procured by locating a female which had just emerged. The place where this pupal case was taken was on a side hill, about 75 feet above the bed of a small stream. The description follows:

"*Pupa.*—[Plate 11, Fig. 126; Plate 13, Fig. 165.] Length 26 mm., diameter 6 mm. Color yellowish brown, the thorax being nearly the same color as the abdomen. Tubercles of the head region well marked and distinctly darker than the surrounding parts. Prothoracic spiracular tubercle brown in color, elevated, narrow, ventral half oblique, dorsal half turned directly forward, thus forming a distinct bend near the middle of the length; rima nearly straight from outer end to the middle and evenly curved for the remainder of its length, inner tip curved backward, thus forming a well-defined hook. First abdominal spiracle nearly round; its rima following the posterior curvature, very narrow, but a little widened above; remaining abdominal spiracles a little smaller than the first one, each with a short, slightly curved or straight rima. Terminal abdominal segment [Plate 13, Fig. 165] with several small spines near the middle of its length and six larger spines at its apex. These spines are all brown in color, with the apex of each approaching black. Six apical spines of nearly the same size; the dorsal pair point upward, outward, and slightly backward, the lateral one on each side outward and backward, while the ventral pair extend almost directly backward. These six spines mark the corners of a hexagon with nearly equal sides, but the ventral pair are a little nearer together than the dorsal pair."

This appears to be all that we know about the early stages of this species. From the place where the pupa was found, the larva appears to be terrestrial in habit, like that of *Tabanus fronto*, but one should bear in mind that possibly the young larva is aquatic and leaves the water at a later stage of its development.

Tabanus tæniola Palisot de Beauvois.—This species is, according to King, the most common and most widely distributed tabanid found in the Anglo-Egyptian Sudan, and the most frequently accused of causing the death of camels. Occurring on the White Nile as far north as Dueim, stray specimens have occasionally been taken in Khartoum. King was able to secure oviposition in captivity in this species as well as in *Tabanus par*, and has described the egg and larva. The pupal stage is not known.

In order to secure eggs, gorged females were taken in May, on cattle grazing near Bor, and placed in a breeding cage with a dish containing grass and weeds growing in mud and water. They were fed on sugar and water, and a few batches of eggs were obtained. A single egg batch was taken in May on a blade of grass overhanging a dried up water pool near Kanissa wood-station, and a number of batches of eggs were collected early in July from grasses and weeds overhanging rain pools at Gebelein.

The eggs (Plate 1, Figs. 8 and 9) are placed by the female fly on the upper side of a blade of grass or some similar plant, and, with the exception of the single batch taken at Kanissa wood-station, all those found were overhanging water. An unfinished batch of eggs resembles an arrow-head. The eggs are closely applied to each other and left bare, so the batch can easily be seen when freshly laid, owing to its shining white to yellowish white color. Prior to hatching, the egg mass becomes darker. According to King, "the egg is spindle-shaped, about 1.75 mm. in length and, when first laid, is white in colour. It becomes darker as the embryo within develops."

The eggs obtained in the breeding cage were laid on May 24 to 25, and hatched on May 29. This would correspond to the very short incubation period of four to five days. The larvæ (Plate 1, Fig. 9, *a*) were placed in glass basins containing mud, growing grass, and water, and were offered the expressed stomach contents of female ticks—*Rhipicephalus simus*—taken from a dog. They fed readily on this until June 11, when they were placed in clean river sand and water, and their diet was changed to mosquito larvæ. These mosquito larvæ were either killed or laid living on the wet sand out of reach of the water, in which position the tabanid larvæ were able to kill them. In water the mosquito larvæ were too active to be caught. On July 16 their food was changed again to freshly killed and bruised earthworms, and these they also ate readily. While still young they became vicious cannibals, and consequently each larva had to be given a separate dish. They were brought to Khartoum on July 19 and a few days later it was noticed that the majority were not taking their food. They were then nearly if not quite full grown, so it was thought that they had buried themselves in the sand prior to pupating. A thorough search, however, revealed the fact that they had disappeared, and it was not until later that mice were identified as the cause of the loss. The two remaining larvæ were then killed and preserved. It is possible, therefore, that the larva described by King is not quite mature.

The larvæ of *Tabanus tæniola* are more active and ferocious than those of *Tabanus par*, vigorously attacking any other larva with which they may come in contact. They have not, however, the power possessed by *Tabanus par* of lying dormant in the soil for at

least fifty-seven days if the conditions are unfavorable for their development.

King describes the larva somewhat as follows:

"The larva [Plate 3, Fig. 49], when fully extended, measures about 29 mm. Color white to grayish white, mandibles black. On the anterior third of each abdominal segment, except the eighth, is a ring of pseudopods, eight in each ring —two dorsal, two lateral, four ventral—except on the first abdominal segment, where the dorsal pair is wanting. On the second abdominal segment the dorsal pair is very strongly developed. The ventral pseudopods[28] are always larger than the dorsal. Each pseudopod bears a crown of colorless spines or hooks, and between the pseudopods there are also spines or hooks, often darker in color, and forming a continuous ring. The anus is situated ventrally at the base of the eighth abdominal segment and is edged with dark hairs. On either side of the anus is a patch of dark hairs, roughly kidney-shaped, and beyond each patch, laterally placed on the segment, are two small round spots of dark hair. The syphon tube consists of two segments, and when exserted is shorter than the eighth segment. The whole surface of the larva is more or less shiny, with varying longitudinal striation, the areas bearing very fine striæ being markedly duller than the rest. The prothorax has the dorsal area smooth in the anterior two-thirds and rather coarsely striate posteriorly; the ventral area is almost entirely smooth and divided in two by a medium furrow; the two lateral areas are finely striated in the basal third and more coarsely so in the anterior parts. The mesothorax has the dorsal and ventral areas smooth and shining in the anterior two-thirds, and rather coarsely striate posteriorly, the ventral area having no furrow; the lateral areas are a little more finely striate than those of the prothorax, and there is a rather broad dull non-striated band at both the anterior and posterior margins. Similar dull bands occur on the metathorax and the abdominal segments, but completely encircling the segments. The abdominal segments 1 to 7 have the dorsal and ventral areas moderately shining, and the striation is rather coarse and irregular; the lateral areas appear much duller, owing to the extreme fineness of the striation. On the eighth abdominal segment the striæ are moderately well marked and of similar appearance on all the faces."

The pupa of *Tabanus tæniola* is not known. From the egg mass a natural enemy parasite was obtained by King, adding hereby one more example to the cases of egg parasitism observed in tabanids. These small Hymenoptera were bred from an egg mass of *Tabanus tæniola*, taken at Gebelein. The species had not yet been identified at the time when King's report appeared, but figures were given, together with the parasitized egg mass, showing the exit hole of the

[28] See p. 108, foot-note 18.

parasites. To judge from the illustration it might be a species of *Phanurus*, but as the figures are life size, not much can be seen of structural details.

As elsewhere in tropical Africa, this is probably the commonest species of the genus in the Mlanje district, where Neave bred it also from larvæ.

The larva is, according to Neave, chiefly remarkable for its white color and lack of pigment, and for the presence of a row of bristles immediately anterior to the anus. It is one of the most active and restless species Neave had to deal with.

Tabanus (*Neotabanus*[24]) *triangulum* Wiedemann.—This Brazilian species was reared by Lutz, at Manguintros, Brazil, in February, 1914, from full grown larvæ, which were obtained by sifting the mud from the edges of a small brook (together with those of *Neotabanus ochrophilus*), and breeding in damp moss. No morphological differences were observed between this species and *Tabanus ochrophilus*.

Tabanus trimaculatus Palisot de Beauvois.—A species occurring in the middle and southern states west to Kansas (Hine). We possess very few data on its early stages.

Hine mentions (1903) that he has been in possession of the eggs, in connection with his studies on extermination of tabanids by collecting and destroying the eggs. By counting it was found that twenty egg masses of *Tabanus trimaculatus* averaged over 500 eggs each.

Brimley found, on April 15, 1909, two larvæ of *Tabanus*, under the bark of a soggy log which was an inch or two above the water. One died; the other was put into a bottle with some wet dirt and rotten wood, and "from this a male of *Tabanus trimaculatus* was bred on May 18 of the same year. The larva that died and which was presumably the same species was preserved in alcohol. It measures 37 mm. in length and is white without markings."[29]

Tabanus tropicus Linné.—A species found in Europe (Laibach and Trieste, according to Schiner), but also in India (same species?) and here said to be a carrier of surra (Neveu-Lemaire).

[29] In the meantime, I have repeatedly found the larvæ in Princeton (1917) and bred the adult.

Scholtz (1850) reports that he has found the pupæ, together with those of *Tabanus autumnalis* and of *Hæmatopota pluvialis* in the neighborhood of Breslau in June, 1850, at the edge of a pond covered with *Lemna*, the water of which was polluted from manure piles surrounding it. The pupæ were found near the edge under a thick mass of moist *Lemna*, together with *Stratiomys*—and *Syrphus*—pupæ;[30] the flies hatched after a few days.

Tabanus ustus Walker.—An African species, observed by Neave in enormous numbers around a pool in a nearly dry stream bed, on the plains in Portuguese territory, southern Nyasaland.

One male and three females were bred in the laboratory at the end of October and beginning of November. The larva, of which the terminal segments are figured (Plate 5, Fig. 66), resembles that of *Tabanus biguttatus*, but is less pigmented.

The upper hooks of the pupal aster are considerably larger than the remainder. The spines of the dorsolateral comb are much reduced, especially in the male.

The pupal aster of the male and the dorsolateral comb of both sexes are figured (Plate 14, Fig. 177, *a*, *b*, *c*).

Tabanus variabilis Loew.—An African species, not rare near Mt. Mlanje, southern Nyasaland, on wooded streams in the neighborhood of the mountain in October and November, occasionally later.

The larvæ were found in this locality by Neave, in abundance. They are entirely different from those of *Tabanus atrimanus*, being almost colorless, though in quite mature individuals the base of the syphon and the syphon itself are of an orange color. The most striking peculiarity of this larva is, however, the presence of a distinct papilla of a dark color on each side of the anal segment. This is easily recognizable in life and distinguishes this species from any other seen by Neave. The anus is also unusually prominent.

The pupa is also remarkable for its dark coloration, especially on the dorsum of the thorax. The aster is characterized by the large horizontally extended middle pair of hooks, and its outline is therefore entirely different from that of the closely allied *Tabanus atri-*

[30] See also pp. 72 and 91.

manus. The dorsolateral comb consists of a few short and rather stout spines.

Figures are given of the syphon of the larva (Plate 5, Fig. 70), the pupa (Plate 12, Fig. 138), pupal aster, and dorsolateral comb of both sexes (Plate 14, Fig. 176, *a–d*).

Tabanus virgo Wiedemann.—A small species, recorded for Bengal and South India. On its early stages we possess notes through the work of Patton and Cragg.

As in all the small tabanids observed by these authors, the eggs are laid on blades of grass just at the edge of a shallow stream, or on the leaves of the lotus plant at the edges of small ponds, but never over deep water. The larvæ, as in the other small species, are said to have no air sacs, and to die when falling into deep water.

The mature larva of *Tabanus virgo* is figured by the same authors (Plate 4, Fig. 63). Attention is called to the openings of the tracheæ being flush with the body. In fact the syphon is extremely short when compared with that of *Tabanus bicallosus*, while as shown in the figures, in *Tabanus ditæniatus* it is of medium length. On the figure given, the larva of *Tabanus virgo* shows no striation or color pattern, but the mouth-parts appear larger than in the other species, and the prolegs are large, fleshy, and somewhat modified.

A small egg mass of this species is figured (Plate 1, Fig. 16) as laid on a dry twig, and consisting of only five eggs, attached to a twig which in fact is much smaller in diameter than the eggs themselves.

The pupa is figured (Plate 11, Fig. 134) by Patton and Cragg, and in addition, an enlarged figure of the eighth abdominal segment of the pupa is given (Plate 12, Fig. 146), showing the six terminal teeth and their arrangement.

Tabanus vivax Osten Sacken.—A North American species which apparently is never very plentiful, but has been taken in a number of the eastern states (New York, Maine, Ohio, etc.). In Ohio it is on the wing during the last half of June.

The life history has been worked out by Hine. The eggs are deposited in masses composed of several hundreds, on stones that project above the water in riffles of streams. The egg mass is nearly round in outline, only slightly convex, composed of about three

layers one above the other. The color of the whole mass is brown, mottled over the top with white. In these respects they do not differ in particular from the eggs of other species of the genus, but the masses observed were not so convex as those of *Tabanus atratus*, and being placed on stones of a color similar to themselves are rather difficult to see. Females have been observed ovipositing as early as June 8, but most often eggs are deposited after this date.

The larvæ (Plate 3, Fig. 43) occur in the streams in the fall. Hine states that in September and October each year they collected the larvæ of the dobson-fly (*Corydalis cornuta* L.) for study in the laboratory. By turning stones at the edge of swift riffles, or by means of a net stretched across the riffles to catch such specimens as are dislodged by turning stones behind the net in the stream, in addition to *Corydalis* larvæ, a large number of the larvæ of this horse-fly were found. Though having done much collecting in streams, Hine asserts that the larva of *Tabanus vivax* is the only tabanid larva taken in riffles so far. It was not found difficult to rear these larvæ. Larvæ taken late in the fall were placed in damp sand and fed on angleworms. As winter approaches they refuse to eat and remain quietly in the sand until the following spring; then they feed actively for a few days and change to pupæ. They reach the adult stage in late spring or early summer. Like other tabanid larvæ, the larvæ of *Tabanus vivax* are not particular as to their food; all that appears to be necessary is that they obtain small soft bodied animals. Crustaceans serve them as well as insects and their own species as well as some other species—whatever, in fact, is in the sand of the breeding cage.

Hine has never observed the larvæ in nature in the spring; consequently their habits at this time of the year are not exactly known, but Hine supposes that they leave the water and pupate in the earth near at hand.

Hine's description of the larva and pupa follows:

"*Larva.*—[Plate 3, Fig. 43.] When full grown, about 25 mm. long. General color yellowish white, anterior margin of each thoracic segment and a narrow band, including the prolegs, on the anterior half of the first seven abdominal segments opaque, and appearing darker than the other parts, which are more or less shining and usually finely striate longitudinally. Prothoracic segment di-

vided by longitudinal grooves into four nearly equal parts, which may be called the dorsal, ventral, and lateral areas. The lateral areas are shining and finely striated on the posterior third and opaque on the anterior two-thirds; the dorsal and ventral areas are opaque on about the anterior fourth and distinctly shining on the remaining parts. The ventral space is plainly divided into two equal parts by a longitudinal groove. In order to see the character of this segment, it must be fully extended. The mesothoracic and metathoracic segments have a number of longitudinal grooves, some of which are very narrowly bordered by opaque darker coloring, which proceeds backward from the narrow anterior border of these segments. Each of the first seven abdominal segments has on its anterior part a transverse row of eight tubercles which encircles the segment. These all bear short spines or claws at the apex, excepting a dorsal pair on each of the first three or four segments. They may be called prolegs, since they have the parts necessary to such organs and, what is more, are used as prolegs. On the posterior dorsal border of most of the abdominal segments there may be a narrow, irregular, opaque marking of the same color of the narrow band in the region of the prolegs; eighth segment on each side with two narrow, curved markings which have the appearance of being composed of contiguous punctures. These markings are of the same shade of color as the other darker areas, and the lower one is more than twice as long as the upper."

"*Pupa.*—[Plate 11, Fig. 128.] 18 mm. long and 4 mm. in diameter. Light brown in color, thorax somewhat paler than the abdomen. Antennal and other tubercles of the head and thorax prominent and darker than the surrounding parts. Prothoracic spiracular tubercle slightly elevated, reniform, oblique; rima uniformly curved for nearly its whole length; but just before the anterior end the curvature is stronger, although no hook is formed. First abdominal spiracle nearly round; rima almost uniformly curved, posteriorly very slightly widened just at the end, anteriorly slightly narrowed and curved so as to form a short hook. The other abdominal spiracles agree with the first one in general, but there is slight variation in the enlargement and curvature of the extreme ends. Terminal teeth [Plate 13, Fig. 160] prominent, shining brown in color, darkest at the extreme tips. Dorsal pair of teeth smallest and closer together than the ventral, lateral teeth longer and larger than the ventral and located much beneath the dorsal, in fact they are nearly midway between the dorsal and ventral."

Tabanus sp. Nos. 1 and 2.—Following Mann's observations, Kollar succeeded in finding freshly laid eggs of another species of *Tabanus*, which was not determined, in a damp meadow at Dornbach near Vienna, of which, after nine days, similar larvæ hatched as were obtained from the egg masses from Wippach, of *Tabanus quatuornotatus*. Kollar also found a parasitic wasp ovipositing in the *Tabanus* eggs; this wasp was found to be specifically different from that obtained from the eggs of *quatuornotatus*.

At about the same time eggs of another undetermined species were found by Hofmann, quoted by Kollar. No descriptions are given.

Tabanus sp. No. 3.—Riley and Johannsen (1915) give a photographic illustration of the egg mass of an American species of *Tabanus*, the name of which is not given (Plate 1, Fig. 6).

Tabanus sp. No. 4.—(Hart's *sp. a.*) Two examples of larvæ of this peculiar species have been collected by Hart, from diverse situations. One was taken under bark in woods near Urbana, Illinois, April 6; the other, from a prairie ditch, in Kane County, Illinois, which was swollen by a heavy rain. Hart's description is as follows:

"*Larva.*—Length 19 mm., diameter 2.5 mm. Last antennal joint short and very slender, epistoma not sulcate anteriorly, but with an elongate puncture. Whitish, lateral pubescent stripes wanting, annuli much reduced and pale except upon false feet. Prothorax shining, with anterior opaque annulus; lateral areas as long as the dorsal, their upper and lower thirds rather coarsely striate, middle third smooth, with several punctures; ventral area smooth, middle groove with three striæ, dorsal area nearly smooth. Striæ of upper lateral spaces of meso- and metathorax and of dorsal and ventral areas of abdomen moderately coarse; those of lateral area of abdomen somewhat finer; dorsal and ventral areas of mesothorax with a few striæ; of metathorax rather sparsely striate. All areas more or less shining. On the anterior side of each dorsal false foot, at its outer end, an opaque light brown elongate fleck. False feet shining and rather finely striated on each side. No projecting spine posteriorly; only a narrow pale annulus on last segment, at base of breathing tube."

Tabanus sp. No. 5.—(Hart's *sp. b.*) Hart reports that, in collecting the larvæ of *Limnophila* and *Bittacomorpha* in the swampy slough of Station I, many small tabanid larvæ were found in the mud and debris, and among them occurred, on April 15, two examples of a distinct very white form with faint markings like those of *stygius*, but laterally striate more like *atratus*, and with a conspicuous isolated smooth spot in the lateral striated area of the prothorax. He describes the larva as follows:

"*Larva.*—[Plate 3, Fig. 42.] Length 22–23 mm., width 2.5 mm. Very pale whitish, markings like those of *stygius*, but pale yellowish fuscous and inconspicuous. Head pale brownish."

"Lateral prothoracic areas not more than half as long as the dorsal, striation scarcely visible, microscopically fine and opaque, much finer than that of the middle and lower lateral areas of the mesothorax, which are somewhat shining; a rather large smooth spot included in the striated lateral area of the prothorax, not far from the shining ventral area but entirely isolated from it; remaining upper lateral thoracic areas distinctly more coarsely striated than the areas below them, and quite shining; abdominal lateral areas more finely striate, feebly shining, microscopically striate on the posterior portion of each area. Dorsal and ventral areas shining, with sparse marginal striæ interrupted on the disks, those of thorax especially smooth."

"Dull annuli broad, including the false feet, a distinct transverse dorsal and ventral pale spot in front of the false feet. Lateral prothoracic area occupied by a very pale fuscous opaque quadrate spot in front of the striated area. Meso- and metathoracic lateral stripes usually visible, but slender, not dilated, lateral edges of dorsal areas diverging; lateral stripes of abdomen almost wanting, except on last two or three segments. Last segment with bases of respiratory tube and anal prominence ringed with opaque fuscous, that around anal prominence sending up an indistinct stripe, with posterior extensions. Pubescence of false feet whitish or pale fuscous. Respiratory tube slender, no spine protruding."

Tabanus sp. No. 6.—Malloch (1917) describes briefly, and without illustrations, an unknown tabanid larva, which differed from other larvæ he had before him, in being entirely white and without lines or patches of pubescence, as well as in being more robust, and less tapered at the extremities. In general appearance it very closely resembled an asilid larvæ, the resemblance being accentuated by the small size of the locomotor organs; and it stood as "Asilidæ" in the Illinois State Laboratory collection. The specimen was obtained in Pulaski, Illinois, June 1, 1910, in a pit-cage used in rearing white

grubs. This is a surprising occurrence, as most of the species of *Tabanus* are confined to damp ground or to aquatic surroundings.

In his analytical key, Malloch gives the following characteristics of this larva, "Body without markings either of color or hairs. Body very closely and finely striated, entirely white." The size of the larva is not given.

Tabanus sp. No. 7.—Unknown European species, observed by Surcouf and Ricardo (1909). Only the pupa is figured (Plate 12, Fig. 150).

Larva.—Surcouf and Ricardo have reported on the capture and rearing of a tabanid larva, of which, however, the species was not determined. Roubaud had given them "a young larva of *Tabanus*" which he had collected on October 3, 1905, at Meudon in the muck of a pond; this larva was reared for eight months in water, feeding not on living prey but on organic matter introduced with a bunch of moss which covered the bottom of the crystallizing dish in which it was kept. On May 13, 1907, it was seeking a protected place in this moss, lost its mobility, turned more translucent than usual, and, on May 16, had transformed into a pupa, which, unfortunately, perished in consequence of an accident. The larva is described as elongate, whitish, very active, pointed at both ends, and provided with a sort of crested ring on each of its segments; these rings are equipped all along their course with retractile tubercles serving as means of locomotion.

Pupa (Plate 12, Fig. 150). The pupa which Surcouf and Ricardo obtained from this larva measured 17 mm. in length and 4 mm. in diameter. This pupa has the aspect, according to the authors, of a lepidopterous pupa; the upper anterior part was smooth above, comprising the thorax and head, the lower anterior parts bearing under their chitinous coverings, the antennæ, the palpi, the eyes, and the first pair of legs. The wings are contained in a case which reaches the top of the scutellar segment, unarmed. The posterior region of the pupa comprises seven segments which have at the tip a crown of rigid hairs intermingling with pointed tubercles with enlarged bases. The last segment has two tubercles, each one consisting of three irregular more or less curved spines. The third thoracic segment and the first six abdominal segments all have a spiracle on each side.

These spiracles appear slightly projecting above the surface.

Tabanus sp. No. 8.—An unidentified African species. No description but good illustration of ventral and dorsal aspect of larva is given by Brumpt (1910). The larva shows dorsal prolegs well developed which indicates an aquatic mode of life (Plate 4, Fig. 55, *a, b, c*).

Tabanus sp. No. 9.—Unidentified African species of which a poor illustration is given by Grünberg (1907), (Plate 4, Fig. 58).

Tabanus sp. No. 10.—A young specimen of an unidentified European species is figured by Graber in his work on the chordotonal organs (1882–83) (Plate 8, Fig. 99); it is supposed to belong to *Tabanus autumnalis*, and may belong to *Chrysops*. (See also page 23.)

Tabanus sp. No. 11.—Henneguy (1904) gives the illustration of a young *Tabanus* larva (Plate 10, Fig. 109), with typically inflated tracheal trunks, in Europe. (See also pages 36 and 38.)

Tabanus sp. No. 12.—Paoli (1907) gives an illustration of the larva (Plate 10, Figs. 111 to 116) in which he studied Graber's organ. Though this larva is supposed to belong either to *Tabanus cordiger* or to *Tabanus autumnalis*, it is not unlikely that it is another species. The two species mentioned happened to be, with *Tabanus bovinus* and *Tabanus quadrinotatus*, and one or two others, the only European species which had been found in the larval state up to that date, but the descriptions are all insufficient.

The larvæ which were studied by Paoli live in slow flowing rivers, especially in the soft mud containing decomposing organic matter, among the algæ, especially *Characeæ*, which grow in the water, in places where they can easily utilize the atmospheric air as they are not provided with organs adapted to locomotion in water.

The body consists of thirteen distinct segments, of which one is the head, three belong to the thorax, and nine to the abdomen; the tenth segment of the abdomen is much reduced and fused with the ninth; the eleventh is absent.

Graber, as Paoli mentions, gives his larva (in the figure) erroneously, only ten segments, while it is known that the larvæ of Diptera have fourteen segments, of which the last one is vestigial.

The largest specimens of the larvæ examined are, when completely extended, 30 to 32 mm. in length. The body is fusiform (Plate 10, Fig. 111), the color a dirty yellowish white, the head is elongate, retractile; the three thoracic segments do not present any peculiarities. The first seven abdominal segments are furnished each one with eight papillæ, arranged all around the anterior third of each segment, which consequently in cross-section presents itself with eight prominences arranged like the corners of a regular octagon. These papillæ are retractile, and each of them is furnished at the tip with numerous strong hooklets. The larva moves in the mud and among the algæ, in addition to making worm-like movements due to the contractions of the subcutaneous muscular stratum, also by protracting and retracting the fifty-six papillæ which in this way form a support and assure to the larva its locomotion. The eighth segment bears on its ventral side a thick sulcate prominence, in the middle of which the opening of the anus is situated. Anterior to this prominence a large lip-like expansion is formed, with numerous strong hooklets which are curved forward at the free margin. Behind the anal opening the eighth segment is narrow and curved upwards; the ninth segment is short, cylindric, thin, more or less retracted into the inside of the eighth, bearing at its extremity the rudiments of the tenth, in which the two stigmata open.

Paoli, after thus briefly describing the larvæ, adds that when disturbed by contact or pressure, they undergo contortions in every direction, making a sound like the crackling of small electric sparks. They feed on animal matter and ferociously attack small larvæ of other aquatic insects, and also devour one another; when they are attacking one another the crackling sounds are heard repeatedly. For the description of Graber's organ in these larvæ see page 29 and following.

Tabanus sp. No. 13.—Maxwell-Leffroy and Howlett (1909) give, on a colored plate, illustrations of the egg mass, young larva, full grown larva, pupa, and an ichneumonid parasite of an unidentified Indian

species of *Tabanus*. The egg mass (Plate 1, Fig. 7) is yellow in the original and not very convex in structure. The young larva (Plate 5, Fig. 76, *a*), is whitish and semitransparent. The full grown larva (Plate 5, Fig. 76, *b*) is yellow, its natural size 47 mm. The pupa is yellow in color, the thorax grayish (Plate 12, Fig. 139). The hymenopterous parasite figured (Plate 5, Fig. 76, *c*) is the only parasite of this (ichneumonid) type observed so far as a tabanid parasite, the other being cases of Proctotrupidæ.

Tabanus sp. No. 14.—Only the pupa of this Indian species (no name given) is figured by Maxwell-Leffroy and Howlett (1909) (Plate 11, Fig. 132).

Eggs and young larvæ described by Lutz (1914) of an unknown Brazilian species which he supposes may belong to the tabanids, probably belong to a leptid species.

PARASITES OF THE EARLY STAGES OF TABANIDÆ.

Hymenopterous Egg Parasites; Early Discoveries.

Kollar (1854) is the first to mention an egg parasite of *Tabanus*. From egg masses of *Tabanus quatuornotatus* collected at Wippach, Austria, by Mann, June 25, 1854, there were observed to hatch not only young *Tabanus* larvæ but "also another completely developed insect belonging to an entirely different order and family, a little animal which belongs to the extraordinarily large and still inextricable army of the parasitic wasps (Ichneumonidæ)." It was concluded that, immediately after the *Tabanus* had deposited its eggs, a parasitic wasp had appeared and deposited its eggs in those of the *Tabanus*. Parasitic wasps were then known to develop in the eggs of Lepidoptera, chiefly Bombycidæ, and of various Hemiptera, but in Diptera such egg parasites had not been observed (Kollar). The length of one of these parasites is given as two-thirds of a line; the size of the *Tabanus* eggs as one line in length, and one-seventh of a line in diameter; Kollar thinks it probable that several wasps develop from a single *Tabanus* egg, which is probably not the case.[31]

On egg masses of a second species of *Tabanus*, collected by himself at Dornbach, near Vienna, Kollar found a similar but specifically different parasitic wasp, which was just ovipositing its eggs in those of the *Tabanus*. In order to learn how much time was necessary for the development of the wasp, Kollar kept the *Tabanus* eggs in a small bottle closed by means of perforated paper, and obtained on the fifteenth day several hundred wasps. From the number of parasites he believed he was justified in assuming that from one *Tabanus* egg more than one wasp may develop. However the number of eggs in the cluster had been said, for *Tabanus quatuornotatus* Meig. to be 350 to 400, and it is not said whether the number of wasps hatching exceeded the number of eggs.

The two sexes of the parasites could be differentiated; a description and figures were promised but apparently have never been published.

[31] See Hart (1895), *Phanurus tabanivorus*, p. 272.

Hart (1895) is the next observer who noticed the egg parasites of Tabanidæ. Two egg masses of *Tabanus atratus* which had already produced larvæ were placed in a dry vial, and a little later it became evident that both masses had been parasitized by Hymenoptera, minute black imagos emerging freely in the vial. An examination of one of the masses showed that about one-half of the eggs had been infested. Examples of the imago were sent to Mr. W. H. Ashmead, who found the species to be a new one; and it was described by him as *Phanurus tabanivorus* (Ashmead). An egg of the *Tabanus* is figured, containing one imago of the parasite.[32]

The description of the parasite by Ashmead is given here:

Phanurus tabanivorus (*n. sp.*, *female*).—"Polished black, impunctate, the head and thorax clothed with a fine sparse pubescence. Head subquadrate, roundly emarginate behind, a little wider than the thorax; eyes oval, faintly pubescent; antennæ eleven-jointed, black, if extended backwards not quite reaching to the apex of thorax, and terminating in a long fusiform five-jointed club, the first joint of which is not quite as wide as the second; ob-trapezoidal, twice as wide as long, the second, third and fourth joints transverse quadrate, a little wider than long; the fifth or last joint conical and a little narrower than the preceding joint; the scape is about as long as the funicle with the pedicel, the latter obconical; joints of funicle a little narrower than the apex of the pedicel, the first joint scarcely longer than thick, the second and third small, transverse moniliform."

"Thorax subovoid, not twice as long as wide, the mesonotum scarcely longer than wide, the scutellum lunate, polished, without pubescence; wings hyaline, ciliated, the cilia on the anterior and posterior margins long, much shorter at apical margin; tegulæ black; venation brown, the marginal vein a little shorter than the stigmal, the latter only slightly thickened at tip, the postmarginal vein very long, fully two and a half times as long as the stigmal; legs fuscous, the trochanters, knees, tips of tibiæ and tarsi honey-yellow or testaceous. Abdomen elongate, pointed fusiform, about twice as long as the head and thorax united, polished, the first segment not longer than wide, with an elevation above at base, the second segment the longest, twice as long as wide at apex, the suture between it and the first striated, the third segment hardly half as long as the second, the fourth about two-thirds the length of the third, the three following forming a cone of which the fifth is very short, its apical margin with a median sinus, the sixth twice as long as the fifth, the seventh very short, scarcely discernible; sheaths of ovipositor a little prominent."

[32] Ashmead's illustrations (Figs. 55 and 56 of his paper) have been omitted.

"*Male.*—Length 0.8 mm. Black, but with the head, prosternum, and legs testaceous; the antennæ twelve-jointed, brown-black, with all the joints of the flagellum, except the pedicel and the last joint, small, moniliform, joints three to five increasing in size but smaller than pedicel, joints six to the penultimate a little wider than long; abdomen not, or only slightly, longer than the head and thorax united, the genitalia long, exserted, curving downwards."

"*Habitat.*—Havana, Illinois."

"Types in collections of the Illinois State Laboratory of Natural History and in my (Ashmead's) collection."

"Described from eleven male and nine female specimens bred from the eggs of a common horse-fly, *Tabanus atratus* Fabr."

Hine (1906) also has observed *Phanurus tabanivorus* and reports that he reared more than a hundred specimens from a single cluster of eggs.

Phanurus tabani Mayr.—In his monograph of the N. A. Proctotrypidæ,[33] Ashmead characterized four species belonging to this genus, none of which, however, are closely allied to *Phanurus tabanivorus*. On the contrary, it appears to resemble more closely three European species described under the genus *Telenomus; viz.*, *Telenomus othus* Hal., *Telenomus laricis* Hal., and *Telenomus tabani* Mayr.

Dr. Gustav Mayr, "in his excellent revision of the European species of *Telenomus*" did not recognize the validity of Thomson's genus *Phanurus*.

Ashmead, however, believes that, as defined in his monograph, the genus can be readily separated from *Telenomus*, although both Haliday and Walker have described *Telenomi* which should now be relegated to *Phanurus;* while Thomson, in his definition of *Telenomus*, has included species that really belong to the genus *Hadronotus* Förster.

These errors, according to Ashmead, probably induced Dr. Mayr to reject the validity of *Phanurus* in his work (cited above).

It is interesting to note that *Phanurus* (*Telenomus*) *tabani* Mayr has habits similar to *Phanurus tabanivorus*, the species having been bred by Brauer from a European horse-fly, *Tabanus sp.*

Phanurus tabanivorus, although evidently related, is somewhat larger in the female sex, smoother, with the joints of the antennæ

[33] Ashmead, N. A. *Proctotrypidæ*, Monograph, pp. 140 and 141.

and the segments of the abdomen relatively different, while the male is much smaller, differently colored, and with the antennal joints totally dissimilar.

Dr. Mayr's species, *Phanurus tabani*, approaches nearest to *Telenomus laricis* Hal.,[34] with which he makes comparison; while *Phanurus tabanivorus* agrees more nearly with *Telenomus othus* Hal., represented on the same plate, Fig. 4 (Mayr's paper).

Phanurus emersoni Girault.—A new *Phanurus* egg parasite of Tabanidæ was discovered by Girault quite recently (1916), *Phanurus emersoni* Girault, and was reared from tabanid eggs at Dallas, Texas. Girault gives descriptions of three allied species.

1. *Phanurus opacus* Howard.—Both sexes are black; the thorax above is subglabrous.

2. *Phanurus floridanus* Ashmead.—The head and thorax are polished, the tibiæ and knees pale brown; segments one and two of abdomen have very short striæ at base. The club is stouter than in *ovivorus*.

3. *Phanurus ovivorus* Ashmead.—The club is slenderer than in the preceding, the tibiæ dark, the thorax above showing faint reticulation, cephalad but mostly glabrous. The first two segments of the abdomen do not have striæ at base, or else these are extremely minute and short. In *flavipes* the vertex and scutellum are uniformly finely reticulate. The species *ovivorus* is very close to *opacus*, if not identical with it.

4. *Phanurus emersoni* Girault.—Female, length 0.90 mm. Black, the wings subhyaline, the venation pale dusky, the tarsi yellow. It differs from *Phanurus opacus* Howard in that the male is varicolored in the latter, and from the female *opacus*, *floridanus*, and *ovivorus* in that the vertex and scutum of the latter are densely reticulated. It differs from *tabanivorus* in that the abdomen of the latter is only somewhat longer than the rest of the body, the third segment is not a fourth the length of the third (second?), the thorax above is reticulated, and the male has the entire thorax honey-yellow, also the antennæ (besides the legs and head as in *tabanivorus*). It is closely related to the female of *ovivorus*, which it resembles. The stigmal

[34] Mayr, Entomological Magazine, ii, Plate XIII, Fig. 2.

vein is nearly twice as long as the marginal, and about half the length of the postmarginal. Funicle 1 is half as long again as it is wide, two-thirds the length of the pedicel, No. 2 a little shorter than No. 1, No. 3 still shorter, No. 4 globular, smallest, No. 5 cup-shaped, No. 6 the same, larger, wider than long, Nos. 7 and 8 subquadrate, No. 9 ovate, longer than wide. Short distinct striæ at base of segment No. 2 of the abdomen.

In the male, Funicles 1 to 3 are somewhat longer than in the female, while Nos. 4 to 9 are moniliform, wider than long, small; the club joint is ovate and as long as Funicle 1 and stouter.

Described from a large number of both sexes reared from tabanid eggs at Dallas, Texas (Bishop).

Types.—Catalogue No. 19,664, U. S. N. M., one male, eight females, on two tags and a slide bearing one male, four females.

Types of *opacus*, *ovivorus*, *floridanus*, and *flavipes* examined.

Telenomus benefactor Crawford.—Patton and Cragg (1913), (page 298), say that *Telenomus benefactor* Crawford is another chalcid which parasitizes the eggs of tabanids in the Sudan.

Telenomus kingi Crawford.—This species has been reared from eggs of *Tabanus kingi*.

Unidentified Parasites Recorded.

Chalcid sp. 1. King (1910) has bred, from an egg mass of *Tabanus tæniola*, taken at Gebelein, numbers of a small Hymenopteron, which, in 1910, had not been identified, but was figured, together with the parasitized egg mass, showing the exit hole of the parasites. As the insects are figured in natural size, not much can be said about structural details (see Plate 1, Fig. 8, and text, p. 170).

Chalcid sp. 2. In Madras, a similar insect, which has not been identified (Patton and Cragg, 1913), regularly destroys large numbers of egg masses of *Tabanus albimedius* and *Tabanus striatus*.

A parasitized egg mass can be recognized, according to Patton and Cragg, by the almost black color which it assumes when the development of the embryos of the Hymenopteron is almost complete.

Chalcid sp. 3. A hymenopterous parasite has also been found to infest the eggs of *Goniops chrysocoma* (McAtee). Consequently the phenomenon seems to be quite general.

Ichneumonid sp. An unidentified ichneumonid parasite of an unidentified Indian species of *Tabanus* is figured (Plate 5, Fig. 76, *c*) by Maxwell-Leffroy and Howlett (1909), apparently the only known instance of a true ichneumonid parasitic in Tabanidæ.[35]

Parasites of the Larvæ.

On parasites of the larvæ we possess only a few remarks by Hart (1895), who found a larva covered with small whitish scales which were possibly the eggs of a parasite. Nothing further is known.[36]

[35] On an egg mass of *Tabanus atratus* F., found in 1915, I noticed a small *Hymenopteron* which I took for an egg parasite and which I found to belong to *Trichogramma*. However, no *Trichogramma* hatched from the egg mass, but instead of it, a large number of the well known *Phanurus*, apparently *tabanivorus* Ashmead.

[36] To these should be added the author's own observation of Nematode parasites which were repeatedly found in larvæ of *Chrysops*.

DISCUSSION OF TABLE II.

Of 91 species data are available;[37] but this is not much if we consider that more than 2,000 species of Tabanidæ have been described, and 76 species have been recorded for the State of New Jersey alone. Moreover, in a number of these 91 species from all parts of the world, we have only very fragmentary data, as for instance in the twelve species of *Chrysops* listed; of five of them we possess only notes on their oviposition; the whole life history of none of them has been worked out. There are also comparatively few data on European species.[38]

From the list it is evident that the majority of Tabanidæ of which the life history is known, are aquatic in habitat: of 91 species noted, 65 are aquatic or probably so, 18 are terrestrial or probably so; of 8 species nothing definite can be said about their habits. The terrestrial habit is then to be considered the exception. It is worthy of note that all the known species of *Chrysops* are aquatic, in as far as the eggs are deposited above water, or the larvæ have been found in the mud. The limit between an aquatic and terrestrial mode of life is, of course, not always very sharp when the habitat is in fact the soft mud at the border of ponds, brooks, and streams, as seems to be usually the case, but most authors agree that the larvæ live in mud saturated with water, an environment which differs physiologically very little from water itself. I have therefore listed all species as aquatic the larvæ of which have been found in the mud near water, or where the eggs are deposited above water. But there is no doubt that some tabanids live in the larval condition at con-

[37] Of two or three of the 91 species discussed here, we have in fact only indications about their breeding habits, no actual knowledge of the stages, so in *Gastroxides*, *Tabanus fuscipes*, and *Tabanus nigerrimus*. Leaving these out, we still have about 88 species on which some data are available.

[38] Recently, I have obtained larval stages of 9 other North American species, bringing the total number of species on which data are known close to 100. These species are: *Chrysops niger*, *obsoletus*, *univittatus*, *Tabanus reinwardtii*, *pumilus*, *orion*, two undetermined species of *Tabanus*, and one *genus incertum*.

siderable distance from water and probably a greater number than is apparent from this table, because terrestrial larvæ will be found much more rarely being less accessible and spread over wider areas. Since the genus *Tabanus* is on the whole phylogenetically the younger, in comparison with *Chrysops*, it would appear that in some species of this genus the larvæ have become secondarily terrestrial, a possibility which is of importance for the understanding of the group as a whole. This may also apply to *Hæmatopota*, a genus absent in Australia and apparently of more recent origin —while retaining a rather primitive wing-pattern—showing aquatic tendencies in the oviposition of some species, yet in its common and widely spread species, *Hæmatopota pluvialis*, completely terrestrial in the larval and pupal stages.

Practically nothing is known, with the exception of the few instances listed, of the early stages of the numerous remaining genera of the family Tabanidæ other than *Chrysops* and *Tabanus* proper, and here is a splendid opportunity especially for workers in tropical countries.

In regard to continents, the data in our possession are distributed as follows:

Africa	29 species.	
North America	24 "	
Europe	21 "	
Asia	15 "	(Two identical with European; *viz.*, African species.)
South America	4 "	
Australia	0 "	

It is seen that Africa ranges highest, and concerning this particular subject, has ceased to be a "dark continent," as compared with North America or Europe.

TABLE II.

Statistical Table of Results on Early Stages.

No.	Species.	Habitat.	Egg.	Larva.	Pupa.	Habits of larva.
1	*Chrysops bimaculosa* Neave	Africa.	—	Neave, 1915	Neave, 1915	Aquatic.
2	" *callidus* O. S.	North America.	Hine, 1903	—	—	" ?
3	" *celer* O. S.	" "	Hine, 1903	—	—	" ?
4	" *dispar* Fabr.	India.	Patton and Cragg, 1914	—	—	" ?
5	" *indus* O. S.	North America.	Hine, 1903	—	—	" ?
6	" *longicornis* Macq	Africa.	—	Neave, 1915	Neave, 1915	"
7	" *magnifica, var. inornata*, Aust.	"	—	Neave, 1915	Neave, 1915	"
8	" *mœchus* O. S.	North America.	Hine, 1899	—	—	" ?
9	" *mœrens* Walk. (*æstuans* v.d.W.)	" "	Hart, 1895 Hine, 1903	—	—	" ?
10	" *relictus* Meig.	Europe.	—	—	Beling, 1882	" ?
11	" *vittatus* Wied.	North America.	—	Hart, 1895	Hart, 1895 Malloch, 1917	" ?
12	" *wellmani* Aust.	Africa.	—	Neave, 1915	Neave, 1915	"
13	*Dorcalœmus fodiens* Aust.	"	—	Neave, 1915	—	" ?
14	*Gastroxides ater* Saunders.	India.	—	Maxwell-Leffroy and Howlett, 1909	—	Terrestrial?
15	*Goniops chrysocoma* O. S.	North America.	Walton, 1908 McAtee, 1910	Walton, 1908 McAtee, 1910	McAtee, 1910	"
16	*Hæmatopota crudelis* Aust.	Africa.	—	Neave, 1915	Neave, 1915	Aquatic ?
17	" *decora* Walk.	"	—	Neave, 1915	Neave, 1915	" ?
18	" *insatiabilis* Aust.	"	—	Neave, 1915	Neave, 1915	" ?
19	" *pluvialis* L.	Europe.	—	Zetterstedt, 1842 Brauer, 1869 Perris, 1870 Beling, 1875	Scholtz, 1850 Brauer, 1869 Perris, 1870 Beling, 1875	Terrestrial.

20	*Hæmatopota sp.* ?.....	India.	Patton and Cragg, 1913	—	—	Aquatic ?
21	" *sp.* ?.....	"	Patton and Cragg, 1913	—	—	" ?
22	*Hexatoma pellucens* L.....	Europe.	— —	Marno, 1868 Brauer, 1863	Marno, 1868 Brauer, 1863	"
23	*Tabanus albimedius* Walk.....	India.	Patton and Cragg, 1913	Patton and Cragg, 1913	—	" ?
24	" *atratus* Fabr.....	North America.	Hart, 1895 Hine, 1906	Walsh, 1864 Riley, 1870 Hart, 1895 Hine, 1906 Malloch, 1917	Walsh, 1864 Riley, 1870 Hart, 1895 Hine, 1906 Malloch, 1917	" ?
25	" *atrimanus* Lw.....	Africa.	—	Neave, 1915	Neave, 1915	"
26	" *autumnalis* L.....	Europe.	(Brauer, 1875) Surcouf and Ricardo, 1907	Brauer and Gőszy, 1851 (Brauer, 1875) Graber, 1878 ? Krauss, 1879 von Friedenfels, 1880	Scholtz, 1850	" ?
27	" *bicallosus* Ric.....	India.	Patton and Cragg, 1913	Patton and Cragg, 1913	Patton and Cragg, 1913	"
28	" *biguttatus* Wied.....	Africa.	King, 1908	King, 1908 Neave, 1915	King, 1908 Neave, 1915	"
29	" *bovinus* L.....	Europe.	—	Degeer, 1760	Degeer, 1760	Terrestrial.
30	" *bromius* L.....	"	—	Beling, 1875	Beling, 1875 Surcouf and Ricardo, 1908	"
31	" *carolinensis* Macq.....	North America.	—	—	Malloch, 1917	— ?

TABLE II—*Continued.*

No.	Species.	Habitat.	Egg.	Larva.	Pupa.	Habits of larva.
32	*Tabanus corax* Lw.	Africa.	Neave, 1915	Neave, 1915	Neave, 1915	Aquatic.
33	" *cordiger* Meig.	Europe.	—	Brauer, 1883 Paoli, 1907 ?	Picard and le Blanc, 1913	Terrestrial.
34	" *costalis* Wied.	North America.	Hine, 1908	Hart, 1895	Hart, 1895	"
35	" *desertus* Walk.	South "	—	Bodkin and Cleare, 1916	Bodkin and Cleare, 1916	Aquatic.
36	" *ditæniatus* Macq.	Africa. India.	Patton and Cragg, 1913	King, 1910 Patton and Cragg, 1913	King, 1910 Patton and Cragg, 1913	"
37	" *epistates* O. S.	North America.	—	—	Malloch, 1917	— ?
38	" *fraternus* Macq.	Africa.	—	Neave, 1915	Neave, 1915	Aquatic.
39	" *fronto* O. S.	North America.	—	Brimley, 1908	Brimley, 1908	Terrestrial.
40	" (*Atylotus*) *fulvus* Meig.	Europe.	—	Sharp, 1899	—	Aquatic.
41	" *fuscipes* Ric.	Africa.	—	Neave, 1915	—	Terrestrial ?
42	" *glaucopis* Meig.	Europe.	—	Wahlberg, 1838?	—	" ?
43	" *gratus* Lw.	Africa.	—	Neave, 1915	Neave, 915	Aquatic.
44	" *hilaris* Walk.	India.	Patton and Cragg, 1913	—	—	" ?
45	" *ignotus* Rossi.	Europe.	Del Guercio, 1913	Del Guercio, 1913	Del Guercio, 1913	"
46	" *insignis* Lw.	Africa.	—	Neave, 1915	Neave, 1915	"
47	" *kingi* Aust.	"	King, 1910	King, 1910	King, 1910	"
48	" *lasiophthalmus* Macq.	North America.	Hine, 1906	Hine, 1906	Hine, 1906	Terrestrial ?
49	" *laverani* Surc.	Africa.	—	Neave, 1915	Neave, 1915	Aquatic.
50	" *lineola* Fabr.	North America.	—	Hart, 1895	Hart, 1895 Malloch, 1917	"

51	*Tabanus maculatissimus* Macq.	Africa.	—	Neave, 1915	Neave, 1915	Aquatic.
52	" *medionotatus* Aust.	"	—	Neave, 1915	Neave, 1915	"
53	" *melanoceros* Wied.	North America.	—	Brimley, 1909	Brimley, 1909	"
54	" *nagamiensis* Cart.	Africa.	—	—	Neave, 1915	— ?
55	" *nigerrimus* Zett.	Europe.	—	—	(Scholtz, 1848?)	Terrestrial ?
56	" *nigrescens* Pal. de Beauv.	North America.	—	Hart, 1895	Hart, 1895 Malloch, 1917	Aquatic.
57	" *obscuripes* Ric.	Africa.	—	—	Neave, 1915	"
58	" *ochrophilus* Lutz.	South America.	—	Lutz, 1914	Lutz, 1914	"
59	" *orientis* Walk.	India.	Baldrey, 1911-12	—	—	— ?
60	" *par* Walk.	Africa.	King, 1910	King, 1910	King, 1910	Aquatic.
61	" *perlinens* Aust.	"	—	Neave, 1915	—	"
62	" *quatuornotatus* Meig.	Europe.	Kollar (Mann), 1854 Lécaillon, 1905, '06, '11	Kollar, 1854 Lécaillon, 1905, '06, '11	—	Terrestrial.
63	" *semisordidus* Walk.	South America.	—	Bodkin and Cleare, 1916	Bodkin and Cleare, 1916	Aquatic.
64	" *solstitialis* Schiner.	Europe.	—	—	Brauer, 1851	" ?
65	" *speciosus* Ric.	India.	Patton and Cragg, 1913	Patton and Cragg, 1913	—	"
66	" *spodopterus* Meig.	Europe.	—	Brauer, 1883	—	Terrestrial.
67	" *striatus* Fabr.	India.	Mitzmain, 1913 Patton and Cragg, 1913 Bainbridge and Fletcher, 1914	Mitzmain, 1913	Mitzmain, 1913	Aquatic.
68	" ***stygius*** Say.	North America.	Hine, 1903	Hart, 1895 Hine, 1903 ('06)	Hart, 1895 Hine, 1903 ('06)	"

TABLE II—*Concluded.*

No.	Species.	Habitat.	Egg.	Larva.	Pupa.	Habits of larva.
69	*Tabanus sulcifrons* Macq.	North America	—	—	Hine, 1906	Terrestrial ?
70	" *tæniola* Pal. de Beauv.	Africa.	King, 1910	King, 1910 Neave, 1915	King, 1910	Aquatic.
71	" *triangulum* Wied.	South America.	—	Lutz, 1914	Lutz, 1914	"
72	" *trimaculatus* Pal. de Beauv.	North "	Hine, 1903	Brimley, 1909	—	"
73	" *tropicus* L.	Europe. India.	—	—	Scholtz, 1850	" ?
74	" *ustus* Walk.	Africa.	—	Neave, 1915	Neave, 1915	"
75	" *variabilis* Lw.	"	—	Neave, 1915	Neave, 1915	"
76	" *virgo* Wied.	India.	Patton and Cragg, 1913	Patton and Cragg, 1913	Patton and Cragg, 1913	"
77	" *vivax* O. S.	North America.	Hine, 1903	Hine, 1903	Hine, 1903	"
78	" *sp.* ? No. 1.	Europe.	Kollar, 1854	Kollar, 1854	—	Terrestrial.
79	" " ? No. 2.	"	Kollar (Hofmann), 1854	—	—	— ?
80	" " ? No. 3.	North America.	Riley and Johannsen, 1915	—	—	— ?
81	" " ? No. 4.	" "	—	Hart, 1895 Malloch, 1917	—	Terrestrial.
82	" " ? No. 5.	" "	—	Hart, 1895 Malloch, 1917	—	Aquatic.
83	" " ? No. 6	North America.	—	Malloch, 1917	—	Terrestrial
84	" " ? No. 7.	Europe.	—	Surcouf and Ricardo, 1909	Surcouf and Ricardo, 1909	Aquatic.
85	" " ? No. 8.	Africa.	—	Brumpt, 1910	—	" ?

86	*Tabanus sp.* ? No. 9.....	Africa.	—	Grünberg, 1907	—	— ?
87	" " ? No. 10.....	Europe.	—	Graber, 1882, '83	—	Aquatic..
88	" " ? No. 11.....	"	—	Henneguy, 1904	—	" ?
89	" " ? No. 12.....	"	—	Paoli, 1907	—	" ?
90	" " ? No. 13.....	India.	Maxwell-Leff-roy and How-lett, 1909	Maxwell-Leff-roy and How-lett, 1909	Maxwell-Leff-roy and How-lett, 1909	" ?
91	" " ? No. 14.....	"	—	—	Maxwell-Leff-roy and How-lett, 1909	— ?

NOTES ON METHODS OF REARING AND STUDYING TABANIDS IN EARLY STAGES.

Collecting the Larvæ.—The natural habitat of tabanid larvæ is the wet mud or sand in the immediate neighborhood of water, hence they are rarely caught by the usual method of collecting aquatic insects by means of a net. The lack of a practical collecting method is probably the main reason why these animals while very common have passed almost unnoticed in an epoch of fresh water biological investigations. Neave seems to have employed large nets, as did Hart also, but Neave was able to send out a collecting staff of negroes to obtain his material. The use of an ordinary sieve, first applied by Hine to collect the larvæ of *Tabanus vivax* in rapid streams, is more advisable for collecting the larvæ from the mud. Patton and Cragg suggest placing lumps of mud and sand with water in a pail; by stirring the mass the large larvæ appear floating at the surface. The smaller ones are caught when the muddy water is passed through a sieve.

Rearing Vessels.—The methods of various authors in rearing tabanid larvæ have been treated in connection with the species. Thus in the paragraphs on *Tabanus lasiophthalmus* and *vivax*, details of Hine's method in rearing these species are found, and Mitzmain's valuable contributions to breeding technique are found under *Tabanus striatus*.

Hine proposes to keep the larvæ in jelly glasses the covers of which are perforated by a few holes, and which are half filled with wet sand. Mitzmain uses dishes with some mud and wet filter paper.

Patton and Cragg have advised the use of very large trays, several feet long and six inches high, in which large numbers of larvæ may be reared under almost natural conditions. The bottom of the tray has to have a hole closed by a cork stopper, to make it possible to change the water in the tray from time to time. Pupæ should be taken out, as the larvæ may injure them, and placed in separate cages, in little vertical holes. Near each pupa a little flag is fastened, bearing the number of the pupa, or other data.

Neave, who was raising many different tabanid species in southern Nyasaland, found that the best receptacles which he could obtain locally were the small basin-shaped vessels made of hard clay by the natives. These were of various sizes, from six inches to a foot in diameter. They were placed separately under cages made of mosquito netting on a wood framework. Neave recovered the larvæ for examination or other purposes merely by washing them out of the mud or sand in which they were placed. This of course requires great care in the case of the smaller species.[39]

Feeding.—While most authors give earthworms as food to the larger larvæ, Neave advises the use of immature larvæ of muscid flies, collected from the carcasses of rats, etc., trapped for the purpose. These larvæ buried themselves at once in the mud, where they were apparently consumed by the tabanid larvæ, which thrived under these conditions. The larger species also greedily attacked the freshly killed bodies of small tadpoles, mollusks, and bits of fish, placed on the surface of the mud, though they were seldom actually seen to do this unless examined at night. It was found in most cases that the tabanid larvæ did best in mud or sand, this point being usually decided by the conditions under which they had been found, which was very wet, but without standing water on the surface.

Transportation.—If it is necessary to transport for any distance larvæ which have not reached the resting stage, it is important that the jars, etc., in which they are placed should contain only wet mud or sand and that there should be no standing water on the surface. Some of Neave's earliest captures, which had to be transported 50 or 60 miles from the Shire River to Mlanje, were nearly all lost from this cause, the larvæ being apparently drowned by the movements of the water on the surface.

Treatment of Pupal Stages.—Neave found that the soil in which the pupæ are kept should be considerably drier than that which suits the larvæ.

Patton and Cragg have proposed to remove the fresh pupæ from the vessels in which the larvæ are kept and to place them vertically

[39] For rearing tabanid larvæ in test-tubes, see Marchand, *J. Econ. Entomol.*, 1917, x, 469–72.

in open holes in sand, where they wriggle themselves soon into the desired position. Each pupa receives a label in the form of a little flag which is fastened in the sand. As soon as a pupa has hatched, it is taken out with its label.

According to Neave, it is difficult to obtain good specimens from bred material, as the imagos begin to fly about and to injure themselves before they are hard enough to be killed. They should therefore be placed in larger cages and kept there until they are sufficiently hardened.

Points Important in Description.—Specific differences in tabanid larvæ are not always easy to detect, especially in those of *Hæmatopota*. They are generally found, according to Neave, in the distribution of the pigmented areas on the last segment around the base of the syphon and the anus. These so called pigmented areas are really areas of pigmented hairs (Waterston) in which are entangled small foreign bodies. Their actual color therefore varies to some extent with that of the medium in which the larvæ have lived. The amount of pigmentation, though not its distribution, also varies with the age of the larvæ.

Hart considers the striation of the lateral areas of the prothorax of great systematic importance in the description of the larvæ.

For practical purposes of description the following points should be noted (Patton and Cragg): In the larvæ, abdominal markings, presence or absence of striæ,[40] characteristics of the pseudopods (prolegs), length of the syphon tube, and structure of the antennæ. In the pupæ, length of the antennal sheath, character of the thoracic spiracle, particularly its inner margin, length of the hairs on the abdomen, structure of the abdominal spiracles, and shape and size of the spines and teeth on the eighth segment.

Mr. James Waterston, of the Imperial British Bureau of Entomology, is quoted by Neave as having suggested the convenient name "aster" for the group of hooks at the termination of the last segment of the pupa. The form of this differs a good deal in the various species, and another characteristic which seems of some specific value is the nature of the uppermost section, often isolated,

[40] Especially on the thoracic segments.

of the series of combs on the anterior part of the last segment; this is called by Neave the dorsolateral comb. It is not always present and varies much in form.

Preserving.—To preserve the larvæ and pupæ, for purposes of dissection, etc., our methods are still undeveloped. Hart[41] (1895) says: "The best results with most larvæ of any size were obtained by heating them in water, not too rapidly, to about 200° F., and setting them aside till cool. A small percentage of acetic acid will prevent the collapsing of very soft larvæ." The principal trouble with this method arises from the expansion of the air within, but a slight inflation is sometimes desirable. This method is not suitable for pupæ generally, and Hart advises the use of 80 per cent alcohol and water for their preservation. Experiments with formalin indicate that it will satisfactorily preserve small and easily penetrable forms.

Malloch (1917) has given further methods for preserving the larvæ and pupæ.

[41] Hart (1895), pp. 159 and 160.

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Plate 1.

Fig. 1. Female of *Chrysops mœrens*, ovipositing on *Typha* leaf. After Hine (photograph from life).

Fig. 2. Egg masses of *Chrysops mœrens*, on *Sparganium* leaf. After Hine (photograph).

Fig. 3. Egg mass of *Tabanus stygius* on *Sagittaria* leaf. After Hine.

Fig. 4. Egg mass of *Tabanus stygius* on *Peltandra* leaf. After Hine.

Fig. 5. Egg mass of *Tabanus stygius* in unusual location. After Hine.

Fig. 6. Egg masses of *Tabanus sp.*? After Riley and Johannsen (photograph).

Fig. 7. Egg mass of *Tabanus sp.*? Natural size 24 mm. After Maxwell-Leffroy and Howlett (colored original yellow).

Fig. 8. Egg mass of *Tabanus tæniola*, with chalcid parasites. After King (colored plate).

Fig. 9. *a*, Egg mass of *Tabanus tæniola*; *b*, young larva. After King (colored plate).

Fig. 10. Egg mass of *Tabanus par*. After King (colored plate).

Fig. 11. Young larva of *Tabanus par*. Natural size 10 mm. After King (colored plate).

Fig. 12. Egg mass of *Tabanus bicallosus* on a blade of grass. After Patton and Cragg.

Fig. 13. Two single eggs of *Tabanus bicallosus*. After Patton and Cragg.

Fig. 14. Egg mass of *Chrysops dispar*, on a blade of grass. After Patton and Cragg.

Fig. 15. Single egg of *Chrysops dispar*. After Patton and Cragg.

Fig. 16. Egg mass of *Tabanus virgo*, on a dry twig. After Patton and Cragg.

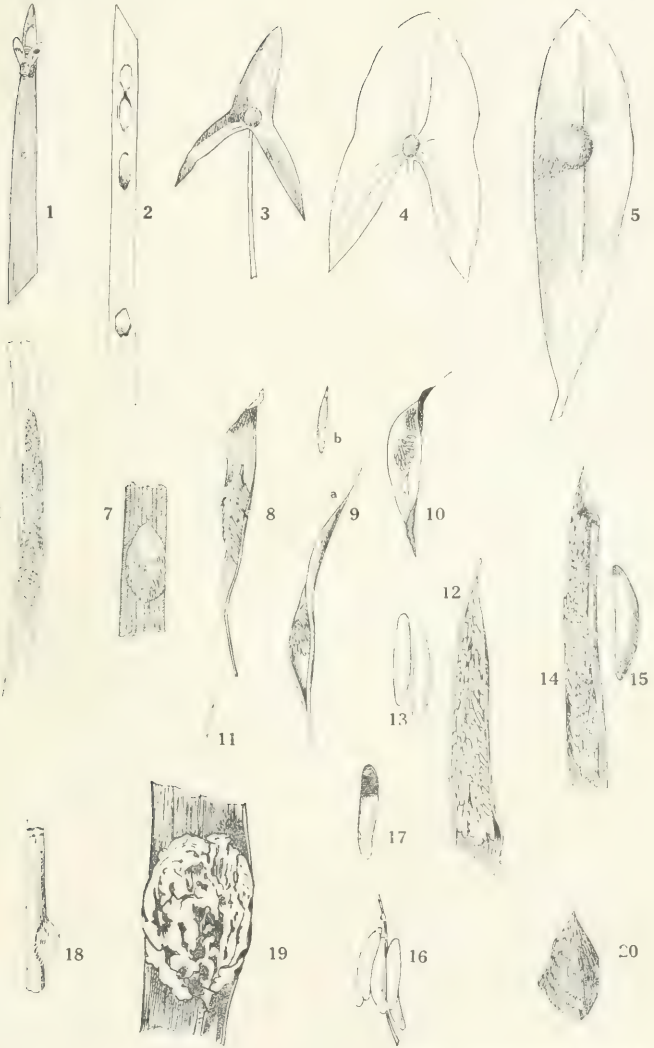
Fig. 17. Single egg of *Tabanus ditæniatus*. After Patton and Cragg.

Fig. 18. Egg mass of *Tabanus biguttatus*. Natural size 14 mm. After King (colored plate).

Fig. 19. Egg mass of *Tabanus speciosus*. After Patton and Cragg.

Fig. 20. Egg mass of *Tabanus kingi*. After King.

King's illustration of a rock situated above water, showing the location of these egg masses, has been omitted.



(Marchand: The early stages of Lebaudia.)

Plate 2.

Fig. 21. Egg mass of *Tabanus diteniatus* spread out in a single layer on a blade of grass. After Patton and Cragg.

Fig. 22. Egg mass of *Tabanus striatus* on paddy leaf. Natural size 13 mm. After Bainbridge and Fletcher.

Fig. 23. Same egg mass, magnified. After Bainbridge and Fletcher.

Fig. 24. Female of *Tabanus striatus*, ovipositing. Natural size of fly 19 mm. After Mitzmain (photograph).

Fig. 25. Egg masses of *Tabanus striatus*. After Mitzmain (photograph).

Fig. 26. Empty (?) egg mass of *Tabanus striatus*. After Mitzmain (photograph).

Fig. 27. Egg mass of *Tabanus striatus* in process of hatching. After Mitzmain (photograph).

Fig. 28. Female of *Goniops chrysocoma*, ovipositing. After McAtee (photograph).

Fig. 29. Egg mass of *Goniops chrysocoma*, seen from above. After Walton.

Fig. 30. Egg mass of *Goniops chrysocoma*, lateral view. After McAtee (photograph).

Fig. 31. Egg mass of *Goniops chrysocoma*. Natural size 14 mm. After McAtee (photograph).

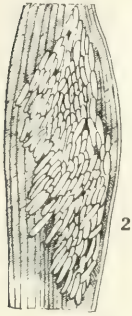
Fig. 32. Empty egg mass of *Goniops chrysocoma* after hatching. After McAtee.

Fig. 33. Double egg mass of *Tabanus quatuornotatus*. After Lécaillon.

Fig. 34. Two single egg masses of *Tabanus quatuornotatus*, Natural size 9 mm. After Lécaillon.

Fig. 35. Group of egg masses of *Tabanus quatuornotatus* on dried twig. After Lécaillon.

Figs. 36, 37, and 38. Egg masses of *Tabanus quatuornotatus*, magnified. After Lécaillon.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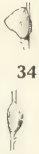
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Plate 3.

Fig. 39. Larva of *Chrysops villatus*. Natural size 15 mm. After Hart (photograph).

Fig. 40. Larva of *Tabanus stygius*. Natural size 52 mm. After Hart (photograph).

Fig. 41. Larva of *Tabanus atratus*. Natural size 53 mm. After Hart (photograph).

Fig. 42. Larva of *Tabanus sp.*? Natural size 24 mm. After Hart (photograph).

Fig. 43. Larva of *Tabanus vivax*. Natural size 36 mm. After Hine.

Fig. 44. Larva of *Tabanus lasiophthalmus*. Natural size 23 mm. After Hine.

Fig. 45. Larva of *Tabanus atratus*. After W. A. Riley and Johannsen (photograph).

Fig. 46. Larva of *Tabanus atratus*. Natural size 52 mm. After C. V. Riley.

Fig. 47. Larva of *Tabanus ditæniatus*. Lateral view of immature larva. After King (colored plate).

Fig. 48. Larva of *Tabanus par*, almost full grown. Natural size 52 mm. After King (colored plate).

Fig. 49. Larva, full grown, of *Tabanus tæniola*. After King (colored plate).

Fig. 50. Larva of *Chrysops wellmani*. Natural size 13 mm. After Neave (Terzi).

Fig. 51. Larva of *Tabanus gratus*. Natural size 23 mm. After Neave. (Terzi).

Fig. 52. Larva of *Tabanus atrimanus*. Natural size 32 mm. After Neave (Terzi).

Fig. 53. Larva of *Tabanus insignis*. Natural size 34 mm. After Neave (Terzi).

Fig. 54. Larva (supposed larva) of *Tabanus pertinens*. Natural size 36 mm. After Neave (Terzi).

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(Marchand: The early stages of Tabanidæ.)

Plate 4.

Fig. 55. Larva of *Tabanus sp.*? *a*, Ventral view; *b*, cross-section of segment; *c*, dorsal view. Natural size 32 mm. After Brumpt.

Fig. 56. Larva of *Tabanus kingi*. After King.

Fig. 57. Larva of *Tabanus ditæniatus*. *a*, Immature larva; *b*, mature larva, lateral view; *c*, 6th and 7th segments of immature larva, lateral view; *d*, caudal end of immature larva. After King.

Fig. 58. Larva of *Tabanus sp.*? After Grünberg.

Fig. 59. Early stages of *Tabanus bovinus*. *a*, Larva; *b*, pupa; *c*, anal end of larva; *d*, anal end of pupa. After Degeer.

Fig. 60. Larva of *Tabanus* (*Atylotus*) *fulvus* (?). *a*, Entire larva; *b*, head and mouth-parts; *c*, one proleg; *d*, segments 10, 11, and 12 in lateral view. After Sharp.

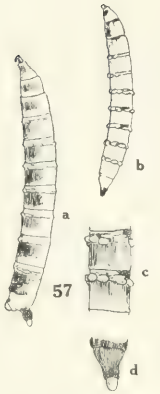
Fig. 61. Mature larva of *Tabanus ditæniatus*. After Patton and Cragg.

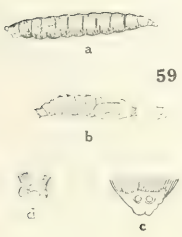
Fig. 62. Mature larva of *Tabanus bicallosus*. After Patton and Cragg.

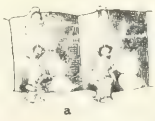
Fig. 63. Mature larva of *Tabanus virgo*. After Patton and Cragg.

Fig. 64. Larva of *Tabanus ditæniatus*. *a*, Segments 3, 4, and 5, lateral view; *b*, segments 11 and 12, lateral view; *c*, segments 11 and 12, ventral view. After King.

Fig. 65. Larva of *Tabanus kingi*. *a*, Lateral view of two abdominal segments; *b*, one proleg; *c*, ventral side of anal segment. After King.







(Matchand: The early stages of Talaniidæ.)

Plate 5.

Fig. 66. Caudal end of larva of *Tabanus ustus*, lateral view. After Neave (Terzi).

Fig. 67. Caudal end of larva of *Tabanus corax*, lateral view. After Neave (Terzi).

Fig. 68. Caudal end of larva of *Tabanus medionotatus*, lateral view. After Neave (Terzi).

Fig. 69. Caudal end of larva of *Tabanus biguttatus*, lateral view. After Neave (Terzi).

Fig. 70. Caudal end of larva of *Tabanus variabilis*, lateral view. After Neave (Terzi).

Fig. 71. Caudal end of larva of *Tabanus maculatissimus*, lateral view. After Neave (Terzi).

Fig. 72. Caudal end of larva of *Chrysops longicornis*, lateral view. After Neave (Terzi).

Fig. 73. Caudal end of *Chrysops*. Dorsal view; diagram. After Neave.

Fig. 74. Caudal end of *Hæmatopota*. Dorsal view; diagram. After Neave.

Fig. 75. *a*, *b*, Larva of *Tabanus biguttatus*. Natural size 42 mm. After King (colored plate).

Fig. 76. *a*, Young larva of *Tabanus sp.?* Colored original greenish white. After Maxwell-Leffroy and Howlett (colored plate). *b*, Full grown larva of *Tabanus sp.?* Same species as Fig. 76, *a*. Colored original yellow. Natural size 47 mm. After Maxwell-Leffroy and Howlett (colored plate). *c*, Hymenopterous parasite of same larva. After Maxwell-Leffroy and Howlett (colored plate).

Fig. 77. Head and first segment of larva of *Tabanus atratus*. After Malloch.

Fig. 78. Mandibles of larva of *Tabanus biguttatus*. After King (colored plate).

Fig. 79. Mandibles of larva of *Tabanus striatus*. After Mitzmain (photograph).

Fig. 80. Larval stages of *Tabanus striatus*. *a*, Newly hatched larvæ; *b*, young larva magnified; *c*, two full grown larvæ. Natural size of the latter 34 mm. After Mitzmain (photograph).

Fig. 81. *a*, *b*, *c*, Shed skins (exuviæ) of three different larval stages of *Tabanus atratus*. After Mitzmain (photograph).

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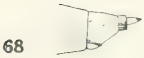
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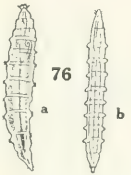
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(Marchand: The early stages of Tabanidæ.)

Plate 6.

Fig. 82. Early stages of *Hæmatopota pluvialis*. *a*, Larva, natural size 13 mm.; *b*, head and mouth-parts, dorsal view; *c*, head and mouth-parts, lateral view; *d*, ventral view of anal segment; *e*, terminal spiracle; *f*, pupa; *g*, pupal structure. After Perris.

Fig. 83. Stages of *Hæmatopota pluvialis*. *a*, Larva, lateral view, natural size 14 mm.; *b*, terminal spiracle of larva; *c*, pupa; *d*, terminal segment of pupa. After Brauer.

Fig. 84. Head and mouth-parts of *Hæmatopota pluvialis*. *ant*, antenna; *lr*, labrum; *lb*, labium; *md*, mandible; *p*, palpus. After Brauer.

Fig. 85. Larva of *Hæmatopota crudelis*. Natural size 13 mm. After Neave (Terzi).

Fig. 86. Caudal end of larva of *Hæmatopota insatiabilis*. After Neave (Terzi).

Fig. 87. Larva of *Tabanus spodopterus*. *a*, Larva magnified; *b*, dorsal view, natural size 45 mm.; *c*, lateral view; *d*, caudal end of larva; *e*, terminal spiracle; *f*, head of larva, lateral view, magnified. After Brauer.

Fig. 88. Larva of *Tabanus cordiger*. *a*, Ventral view of larva, natural size 35 mm.; *b*, caudal end; *c*, cross-section through segment; *d*, caudal end with tracheæ.

Fig. 89. Larva of *Hexatoma pellucens*. *a*, Dorsal view of larva; *b*, dorsal view of last three segments of larva, showing pigmentation. Natural size 35 mm. After Brauer.

Fig. 90. Head and mouth-parts of larva of *Hexatoma pellucens*. *a*, Lateral view; *b*, lateral view of mouth-parts; *c*, dorsal view of head. *lr*, labrum; *md*, mandible; *p*, palpus; *ant*, antenna; *oc*, eye-spot; *lb*, labium. After Brauer.

Fig. 91. Head of larva of *Hæmatopota pluvialis*, dorsal view. Parts as in Figs. 84 and 90. After Brauer.

(Marchand: The early stages of Tabanidæ.)

Plate 7.

Fig. 92. Full grown larva of *Goniops chrysocoma*. *a*, Dorsal; *b*, ventral *c*, lateral view. Natural size 17 mm. After McAtee (photograph).

Fig. 93. Young larva of *Goniops chrysocoma*. *a*, Entire larva, dorsal view; *b*, mouth-parts, magnified, from above; *c*, mouth-parts, lateral view. After Walton.

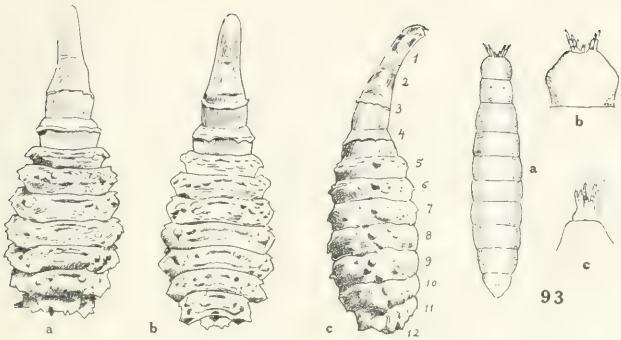
Fig. 94. Full grown larva of *Goniops chrysocoma*. After McAtee (photograph).

Fig. 95. *a*, *b*, Structures of head and mouth-parts of the full grown larva of *Goniops chrysocoma*. *anf*, base of antennæ, *lbr*, labrum; *mx*, maxilla; *mxp*, maxilla palpus. Other details as in Figs. 84, 90, 91. After McAtee.

Fig. 96. *a*, Larva of *Tipula oleracea* and *b*, *Tabanus ignotus*?, drawn for comparison. After del Guercio. (To judge from the figures, both drawings seem to represent tipulid larvæ.)

Fig. 97. Head of *Tabanus albimedius*, lateral view. *md*, mandible; *mx*, maxilla; *ant*, antenna; *sp*, spines; *lr*, labrum; *p*, palpus. After Patton and Cragg.

Fig. 98. Intestinal tract of the full grown larva of *Tabanus albimedius*. *æs*, Esophagus; *pv*, proventriculus; *slg*, salivary glands; *mg*, midgut; *mpt*, Malpighian tubes. After Patton and Cragg.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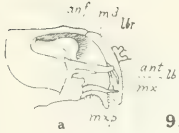
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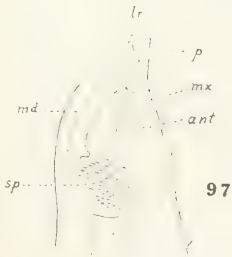
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Plate 8.

Fig. 99. Newly hatched larva of *Tabanus autumnalis* (?). *s* 1 to *s* 13, somites (segments) of the body; *w*, prolegs armed with hooks; *tb*, touch-tactile bristle; *ph*, pharynx; *md*, midgut; *ed*, hindgut; *ma*, Malpighian vessels; *a*, anus; *tr*, tracheal trunks; *t sp*, terminal spine; *g sup*. upper, *g inf*, lower cephalic ganglion; *bg* 1 to *bg* 11, ventral chain of ganglia; *ch* 1 to *ch* 3, chordotonal organs; *ot*, otocyst-like organs on posterior border of anal segment (Graber's organ). After Graber.

Fig. 100. *a*, Right side of 2nd and 3rd body segments of young *Tabanus* larva highly magnified.

2nd segment: *cu*, cuticle; *ma*, matrix; *m*, 1, circular muscles of pharynx; *m* 2, muscles originating on the posterior border of the first segment, and extending forward and inwards, possessing strongly marked transverse striation and great contractility; *hn*, nerve of the skin; *ekg*, *mkg*, ganglion cells connected with this nerve and possessing one nucleus *ekg*, or several *mkg*; *tb*, tactile bristle; s 2 *ch* 1 monoscolopic, s 2 *ch* 2 triscolopic chordotonal organ; *li*, ligament; *st* 2, rods; *efs*, terminal filament; *e* 1, *e* 2, *e* 3, attachments of filaments; *mn*., nerve going to muscle; *tr*, branch of tracheal system; *hg*, ganglion; *nu*, nuclei.

3rd segment, *m* 3, system of sagittal muscles, freely anastomizing, and in fresh condition smooth (unstriated) in appearance; *bl*, region of integument covered with bristles, in anterior part of segment; *s* 3 *ch* 1 to *s* 3 *ch* 6 chordotonal organs (taken from different individuals); (*ch* 1 and *ch 2*, and again *ch* 4, 5, and 6 were observed in the same individual); *e* 4, *e* 5, *e* 6, points of attachments of the terminal filaments of *s* 3 *ch* 5; *e* 7, *e* 8, *e* 9, those of *s* 3 *ch* 2; *g inf*, lower pharyngeal ganglion. After Graber. Zeiss oil immersion lens.

Fig. 100. *b*, Tactile bristle of young larva of *Tabanus autumnalis* (?), with its ganglion. *cu*, cuticle; *tb*, tactile bristle, articulating on its place of attachment; *ma*, matrix (hypoderm), in fresh condition showing no cell limits, with reddish nuclei; starting from it a fine filament torn out of the sheath surrounding the bristle; *k*, nuclei; *n*, nerve; *g*, ganglion. After Graber. Zeiss oil immersion lens.

Fig. 101. Triscolop chordotonal organ of the 2nd segment of the same larva. *n*, nerve; *g* 1, *g* 2, *g* 3, the three ganglion cells, lying one above the other, with reddish nuclei; *xfs*, neurit; *st*, rods; *ko*, heads of rods; *mk*, nucleus of the proximal end; *dk*, of the distal end of the rod; *fs* 1 to *fs* 3, the three terminal filaments. After Graber. Zeiss oil immersion.

Fig. 102. Monoscolop chordotonal organ of the 2nd segment of the same larva *xfs* neurit visible in the interior of the rod. After Graber. Zeiss oil immersion.

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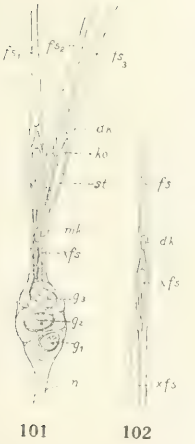

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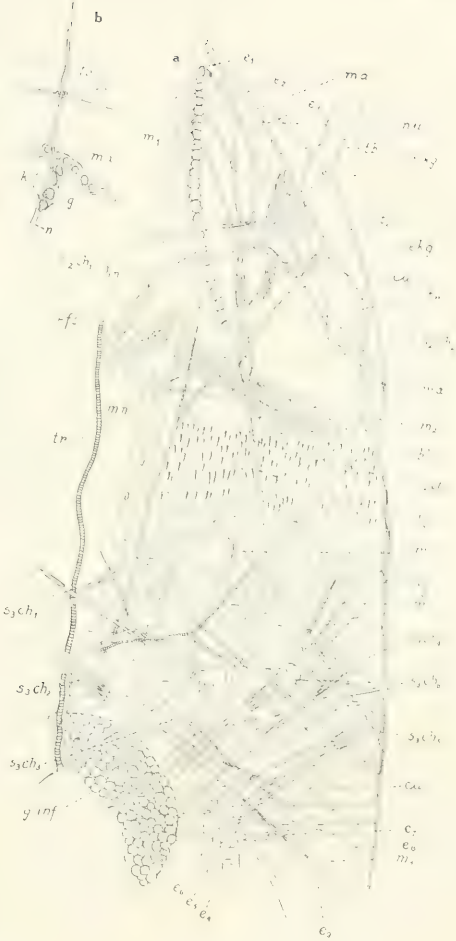


Plate 9.

Fig. 103. Larva of *Tabanus autumnalis* (?). *g*, Graber's organ. After Graber.

Fig. 104. Posterior end of larva, magnified, comprising 9th, 10th, 11th, and 12th segments. (In the original the numbers are given erroneously as 8, 9, 10, and 11.) *tr*, tracheal trunks; *dv*, dorsal vessel; *g*, Graber's organ; *t*, terminal tube; *m*, two muscles extending forward from Graber's organ. After Graber.

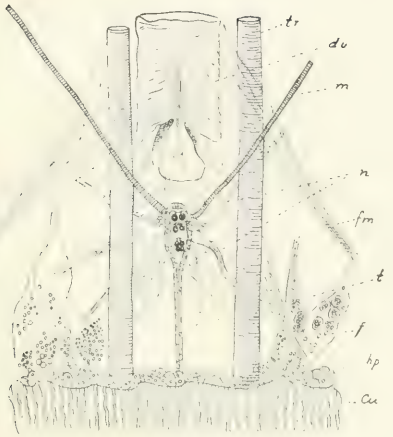
Fig. 105. Graber's organ *in situ*. *dv*, dorsal vessel; *tr*, lateral tracheal trunks; *fm*, muscle of dorsal vessel; *m*, muscles; *t*, terminal tube; *n*, nerves (?); *f*, fat body; *cu*, cuticle of integument; *hp*, hypoderm. × 60. Zeiss oil immersion. After Graber.

Fig. 106. The organ isolated. *c* 1 to *c* 3, chitinous capsules; *sp*, extremity of capsule; *ep*, its epithelium; *cp* 1 to *cp* 4, pedunculate bodies, (in the original only the first capsule bears this name, while the inner ones are treated as "internal sacs"); *m* 1 to *m* 2, muscles; *n* to *n* 1, nerves (?); *ga*, ganglion-like swelling of first nerve; *t*, terminal tube; *op*, operculum. × 133. Zeiss oil immersion. After Graber.

Fig. 107. One pair of pedunculate bodies. *cp*, body; *st*, hollow stem or peduncle; *cu*, scaly cuticle to which they are fastened. × 333. Zeiss oil immersion. After Graber.

Fig. 108. A portion of elastic connective tissue from the surroundings of the organ, after treatment with boiling KOH. × 333. Zeiss oil immersion. After Graber.

Graber's figures, Figs. 103 to 108, were drawn from fresh material.



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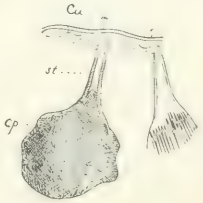

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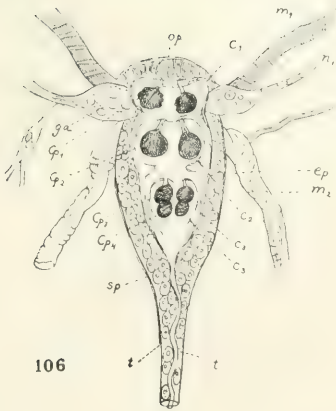

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(Marchand: The early stages of Tabanidæ.)

Plate 10.

Fig. 109. Young larva of *Tabanus sp.*? *tr*, tracheal trunk; *g*, Graber's organ. After Henneguy.

Fig. 110. Graber's organ, enlarged, of larva figured in Fig. 109. After Henneguy.

Fig. 111. Larva of *Tabanus sp.*?, showing the location of Graber's organ (the latter drawn larger than in proportion to the size of the larva). After Paoli.

Fig. 112. Graber's organ in full grown larva of *Tabanus sp.*?, with muscle and nerves attached to it. *c* 1 to *c* 7, capsules; *cp*, pedunculate bodies; *m*, muscle; *n*, nerve. After Paoli.

Fig. 113. Hypothetical diagram of the development of Graber's organ. *c* 1, first capsule; *c* 2, second capsule; *hp*, hypoderm; *ct*, cuticle. After Paoli.

Fig. 114. Terminal tube of Graber's organ. *hp*, hypoderm. After Paoli.

Fig. 115. Last three segments of larva, showing Graber's organ *in situ*. Dorsal views *tr*, tracheal trunks; *m* 1, muscles of the first pair; *a*, anal tubercle. After Paoli.

Fig. 116. Last three segments of larva, with Graber's organ. Lateral view. *m* 2, muscle of the second pair; *t*, terminal tube. Other details as in Fig. 115. After Paoli.

Fig. 117. Young larva of *Tabanus quatuornotatus*. After Lécaillon.

Figs. 118 and 119. Posterior end of body of larva of *Tabanus quatuornotatus*, somewhat older than that figured in Fig. 117, showing the pedunculate bodies being expelled through the terminal tube. After Lécaillon.

Fig. 120. Dorsal view of Segments 11 and 12 (syphon) of a young larva *Tabanus corax*, showing Graber's organ; the dotted lines show the position of the anus. After Neave.

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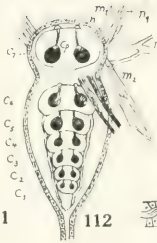

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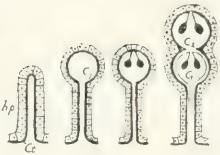

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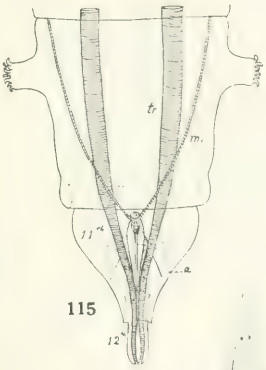

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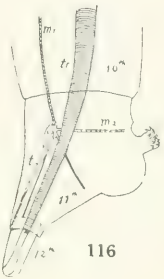

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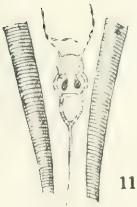

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Plate 11.

Fig. 121. Pupa of *Goniops chrysocoma*. *a*, Dorsal; *b*, ventral; *c*, lateral view. After McAtee.

Fig. 122. Pupa of *Tipula oleracea*. After Paoli.

Fig. 123. Pupa of *Tabanus ignotus*. Compare with Fig. 122. After Paoli. (See also Fig. 96, *a*, *b*. No resemblance is found in the pupæ which indicates that Fig. 96, *b*, does not represent a tabanid larva.)

Fig. 124. Pupa of *Tabanus atratus*. Natural size 31 mm. After Hine.

Fig. 125. Pupa of *Tabanus stygius*. Natural size 28 mm. After Hine.

Fig. 126. Pupa of *Tabanus sulcifrons*. Natural size 27 mm. After Hine.

Fig. 127. Pupa of *Tabanus lasiophthalmus*. Natural size 23 mm. After Hine.

Fig. 128. Pupa of *Tabanus vivax*. Natural size 23 mm. After Hine.

Fig. 129. Pupa of *Tabanus ditæniatus*. After King.

Fig. 130. Pupa of *Chrysops longicornis*. Natural size 11 mm. After Neave (Terzi).

Fig. 131. Pupa of *Hæmatopota insatiabilis*. Natural size 11 mm. After Neave (Terzi).

Fig. 132. Pupa of *Tabanus sp.?* Natural size 18 mm. After Maxwell-Leffroy and Howlett.

Fig. 133. Pupa of *Tabanus ditæniatus*. Ventral view. After Patton and Cragg.

Fig. 134. Pupa of *Tabanus virgo*. Ventral view. After Patton and Cragg.

Fig. 135. Pupa of *Tabanus bicallosus*. Ventral view. After Patton and Cragg.

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Plate 12.

Fig. 136. Pupa of *Tabanus cordiger*. After Picard and le Blanc (from pupal shell).

Fig. 137. *a*, Pupa of *Tabanus kingi; b*, caudal end, lateral view; *c*, caudal end, ventral view. After King.

Fig. 138. Pupa of *Tabanus variabilis*. Natural size 20 mm. After Neave (Terzi).

Fig. 139. Pupa of *Tabanus sp.?* (see Figs. 7 and 76). Colors of original yellow, thorax grayish. Natural size 21 mm. After Maxwell-Leffroy and Howlett (colored plate).

Fig. 140. Pupa shell of *Tabanus atratus*. Natural size 31 mm. After C. V. Riley.

Fig. 141. Caudal end of pupa of *Tabanus ditæniatus*. *a*, Lateral view; *b*, ventral view. After King.

Fig. 142. Pupa of *Tabanus par*. *a*, Entire pupa, lateral view, natural size 22 mm.; *b*, pupal aster. After King (colored plate).

Fig. 143. Pupal aster of *Tabanus striatus*. *a*, Pupal aster of male; *b*, pupal aster of female. After Mitzmain (photograph).

Fig. 144. Pupal aster of *Tabanus bicallosus* ♂. After Patton and Cragg.

Fig. 145. Pupal aster of *Tabanus bicallosus* ♀. After Patton and Cragg.

Fig. 146. Pupal aster of *Tabanus virgo*. After Patton and Cragg.

Fig. 147. Pupal aster of *Tabanus ditæniatus*. After Patton and Cragg.

Fig. 148. Pupal aster of *Tabanus biguttatus*. After King (colored plate).

Fig. 149. Pupa of *Tabanus lineola*, dorsal view. After Malloch.

Fig. 150. Pupa of *Tabanus sp.?* natural size 17 mm. After Surcouf and Ricardo.

Fig. 151. Pupa of *Tabanus bromius* ♂ in process of hatching, natural size 23 mm. After Surcouf and Ricardo.

Fig. 152. Pupa of *Tabanus striatus*. *a*, Lateral view of pupa; *b*, pupa in process of hatching. After Mitzmain.

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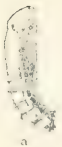

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Plate 13.

Fig. 153. Upper half of thorax of pupa of *Tabanus stygius*, dorsal view. After Malloch.

Fig. 154. Upper half of thorax of pupa of *Chrysops vittatus*, dorsal view. After Malloch.

Fig. 155. Thorax of pupa of *Tabanus cordiger*, dorsal view. After Picard and le Blanc.

Fig. 156. Prothoracic spiracle of pupa of *Tabanus nigrescens*, dorsal view. After Malloch.

Fig. 157. Prothoracic spiracle of pupa of *Chrysops vittatus*, dorsal view. After Malloch.

Fig. 158. Pupal aster of *Tabanus cordiger*. After Picard and le Blanc.

Fig. 159. Pupal aster of *Tabanus lasiophthalmus*. After Hine.

Fig. 160. Pupal aster of *Tabanus vivax*. After Hine.

Fig. 161. Pupal aster of *Tabanus stygius*. After Hine.

Fig. 162. Pupal aster of *Tabanus lineola*. After Hart (photograph).

Fig. 163. Pupal aster of *Tabanus atratus*. After Hart (photograph).

Fig. 164. Pupal aster of *Tabanus atratus*. After Hine.

Fig. 165. Pupal aster of *Tabanus sulcifrons*. After Hine.

Fig. 166. Pupal asters of *Chrysops magnifica, var. inornata*. *a*, Male; *b*, female. After Neave (Terzi).

Fig. 167. Pupal aster of *Chrysops bimaculosa*. *a*, Male; *b*, female; *c*, female, side view. After Neave (Terzi).

Fig. 168. Pupal aster of *Chrysops wellmani*. *a*, ♂; *b*, ♀. After Neave.

Fig. 169. *a*, Pupal aster and *b*, dorsolateral comb of *Hæmatopota decora* ♀. After Neave.

Fig. 170. Pupal aster of *Hæmatopota crudelis*. After Neave.

Fig. 171. Pupal aster of *Chrysops longicornis*. *a*, ♂; *b*, ♀. After Neave.

Fig. 172. Pupal aster of *Hæmatopota insatiabilis* ♀. *a*, From behind; *b*, side view; *c*, dorsolateral comb. After Neave.

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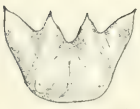
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Plate 14.

Fig. 173. *Tabanus atrimanus*. *a*, Pupal aster of ♂; *b*, dorsolateral comb of ♀; *c*, pupal aster of ♀. After Neave (Terzi).

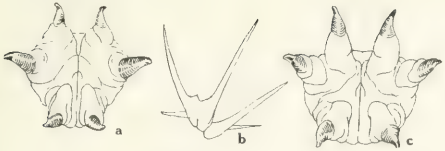
Fig. 174. *Tabanus medionotatus* ♂. *a*, Pupal aster; *b*, profile of last segment of pupa. After Neave (Terzi).

Fig. 175. *Tabanus biguttatus*. *a*, Pupal aster of ♂ and *b*, of ♀; *c*, dorsolateral comb of ♂ and *d*, of ♀. After Neave (Terzi).

Fig. 176. *Tabanus variabilis*. *a*, Pupal aster of ♂; *b*, dorsolateral comb of ♂; *c*, dorsolateral comb of ♀; *d*, pupal aster of ♀. After Neave (Terzi).

Fig. 177. *Tabanus ustus*. *a*, Dorsolateral comb of ♂; *b*, pupal aster of ♂; *c*, dorsolateral comb of ♀ (with six spines). After Neave (Terzi).

Fig. 178. *Tabanus maculatissimus*. *a* and *b*, dorsolateral comb of two different ♂; *c*, pupal aster of ♀; *d*, dorsolateral comb of ♀. After Neave (Terzi).



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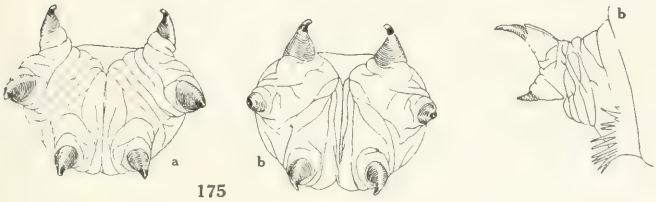

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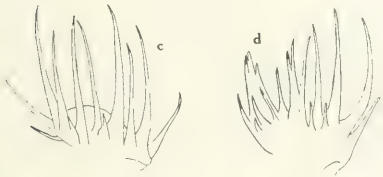

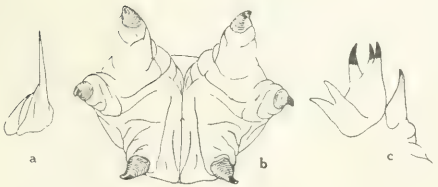

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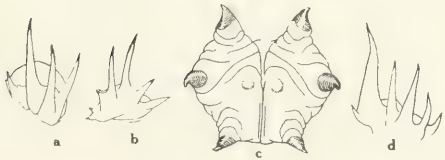

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(Marchand: The early stages of Tabanidæ.)

Plate 15.

Fig. 179. *Tabanus nagamiensis* ♂. *a*, Pupal aster; *b*, pupal aster from the side, showing combs, the dorsolateral comb being absent. After Neave (Terzi).

Fig. 180. *a*, Pupal aster of *Tabanus desertus; b*, lateral view. After Bodkin and Cleare (Terzi).

Fig. 181. *Tabanus corax*. *a*, Pupal aster of ♂; *b*, pupal aster of ♀; *c*, dorsolateral comb of ♀. After Neave (Terzi).

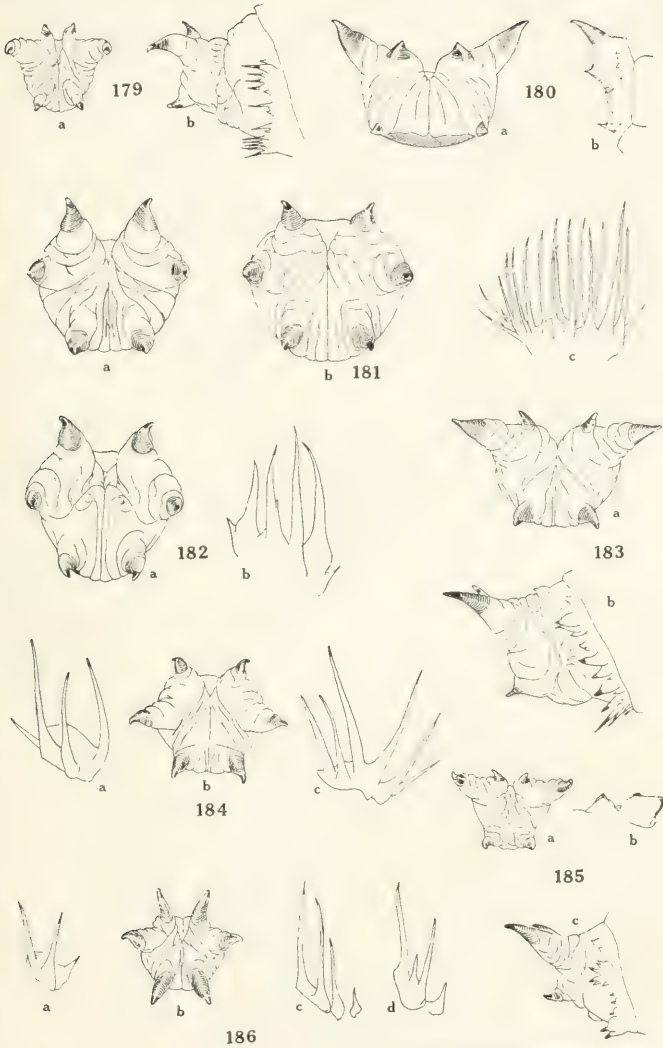
Fig. 182. *Tabanus fraternus* ♀. *a*, Pupal aster; *b*, dorsolateral comb. After Neave (Terzi).

Fig. 183. *Tabanus obscuripes* ♂. *a*, Pupal aster; *b*, profile of last segment of pupa. After Neave (Terzi).

Fig. 184. *Tabanus insignis*. *a*, Dorsolateral comb of ♂; *b*, pupal aster of ♂; *c*, dorsalateral comb of ♀. After Neave (Terzi).

Fig. 185. *Tabanus laverani* ♀. *a*, Pupal aster; *b*, enlarged view of dorsolateral comb; *c*, pupal aster from the side, showing the combs and the small dorsolateral comb. After Neave (Terzi).

Fig. 186. *Tabanus gratus*. *a*, Dorsolateral comb of ♂; *b*, pupal aster of ♂; *c* and *d*, dorsolateral comb of two different ♀. After Neave (Terzi).



Marchand: The early stages of Tabanidæ.

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FURTHER STUDIES ON THE ETIOLOGICAL RÔLE OF VIBRIO FETUS.*

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(Received for publication, July 8, 1920.)

Among the twenty-six cases of abortion associated with *Vibrio fetus*,[1, 2] of which one also contained *Bacillus abortus*, there was none involving heifers. All were second or later pregnancies. All but three were purchased cows. More recently three cases of abortion in native heifers have been found associated with *Vibrio fetus*. These cases are of sufficient importance to be given in detail.

No. 433.—Native heifer; aborted October 24, 1919. Records state that she had been bred February 22, 1919. Male fetus, 28 inches long; color black and white. Coat of hair still lacking. The stomach fluids contained a few particles of meconium only. The contents of the large intestine were normal. The lungs were free from air. There were some small hemorrhages in the auriculoventricular valves. Besides a general fullness of blood in the organs there were no marked abnormalities in the fetus.

Cultures were made as follows: From the contents of the fourth stomach, which showed spirals in films, pure cultures of spirilla were obtained. Films from small and large intestines were negative, but cultures developed spirilla from contents of the colon and rectum. Cultures from the lungs and spleen also contained spirilla only. Those from the liver and kidneys remained sterile.

One guinea pig was inoculated with the contents of the fourth stomach, another with meconium, and a third with lung tissue. Killed after 7 weeks, none showed any signs of disease due to *B. abortus*, and the spleen cultures remained sterile.

A portion of the placenta representing the unoccupied horn, which had passed out, was cut from the still adherent and retained remainder. It was covered with shavings and without any odors of decomposition. During the washing to

* The tests relating to the milk were made by Miss Marion L. Orcutt.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

remove adherent bedding, yellowish, cheesy particles were carried off by the wash water from the cotyledons.

The chorion varies from a smooth, translucent, slightly injected membrane to one opaque, thickened, and leathery. The opacity is due to an infiltration which is in the form of slightly elevated plaques not removable by gentle scraping. In other places the infiltration is discrete, in the form of whitish opacities, $\frac{1}{2}$ to 1 mm. in diameter. There are also scattering minute tufts of adventitious villi, completely cheesy. Edema of the subjacent tissue varies in thickness from place to place. The cotyledons are in part normal, in part diseased. Some of the latter are yellowish, pultaceous throughout. The still normal cotyledons contain, usually on the margin, necrotic, yellowish villi. More rarely such villi are scattered through the cotyledon.

Many films were examined without showing any bacilli of the type of *B. abortus*. There were, however, occasional larger rods and vibrios. A few were closely wound, the rest flatter, as is usual with *V. fetus*.

Sections of fixed and hardened material from various regions of the placenta show necrosis of villi, loss of surface epithelium with infiltration of the underlying tissue with leucocytes in certain areas. Where epithelium is present, no bacteria are found in them, as is the case when *B. abortus* is present. The endothelium of the capillaries has proliferated in places and it partly or nearly fills the lumina. Bacteria resembling vibrios are detected within these cells and in groups among necrotic villi. Inoculation of two guinea pigs with scrapings from the diseased placenta was negative as regards *B. abortus*.

Milk collected 3 days after the expulsion of the fetus, centrifuged according to the method previously described,[3] and injected into three guinea pigs also yielded negative results as regards *B. abortus*. Agglutinins for *B. abortus* were absent from the milk at this time.

The blood agglutinations of this heifer are significant in that they indicate subsequent infection with *B. abortus* during the second pregnancy. 2 days before abortion, October 22, 1919, the titer limit was 1:20, 20 days after abortion a trifle above 1:40. Nearly 6 months later samples taken 4 days apart were 1:640.

No. 438.—Date of breeding uncertain. Aborted November 9, 1919. The placenta was retained. Fetus, female, measures $17\frac{1}{2}$ inches and weighs about 5 pounds. No subcutaneous edema or serous effusions. Autolytic changes of organs without any distinctly putrefactive odors present. The fourth stomach contains a thick mucoid pinkish fluid and films show spirilla. Films of contents of colon and rectum do not show any bacteria. One ventral lobe of the lungs shows under pleura fine, branching, grayish yellow lines made up of masses of spherical crystals.

Cultures from contents of the fourth stomach contain only spirilla. The same is true of cultures of the colon contents. One culture from the rectum contains

[3] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.

cocci, the other only spirilla. Cultures made with bits of spleen, liver, and lungs develop only spirilla. One tube from the kidney contains large bacilli, the other only spirilla.

Four guinea pigs, inoculated respectively with a suspension of material from a uterine swab, with fourth stomach contents, meconium, and lung tissue, were negative as to *B. abortus* when killed and cultured after 6 to 7 weeks.

A sample of milk drawn 9 days after expulsion of the fetus was inoculated into three guinea pigs. *B. abortus* was not isolated from any of them. The titer limit of the agglutinations for *B. abortus* of this heifer 4 days after abortion was 1:40, the milk titer was negative when tested 9 days after. 5 months after abortion the titer limit of the blood was 1:80, of the milk below 1:20.

No. 449.—Aborted during the night of December 1 to 2, 1919. Date of breeding unknown. Female fetus, 16 inches long. Lower jaw and part of upper jaw eaten away by some animal during the night. Some coils of the small intestine protruding from the opening at the umbilicus. General suffusion of tissues with hemoglobin. Autolytic changes indicated by appearance of tissues. Skin readily peeled away from subjacent muscular tissue.

Contents of the fourth stomach turbid. They contain epithelial growths from amnion, swallowed by the fetus. Films from the fourth stomach contents contain numerous spirilla. One culture from the contents contains only spirilla. In the other they are associated with bacilli. Cultures from the liver contain only spirilla. Those from the lungs contain a mixture of rods and cocci.

Only one guinea pig was inoculated with the contents of the fourth stomach. The result was negative as to *B. abortus*.

Milk collected 1 day after expulsion of the fetus was tested on three guinea pigs for *B. abortus* with negative result.

The agglutination titer of this heifer also indicated absence of *B. abortus*. On February 12, 1919, and April 1, it was 1:80. On November 25 it was 1:20. On the date of abortion, December 2, it was 1:40. $4\frac{1}{2}$ months later it was 1:80. The agglutination titer limit of the milk 1 day after abortion was 1:40, $4\frac{1}{2}$ months later it was below 1:20.

Agglutination Tests.

It was stated in an earlier publication[4] that the agglutinability of recently isolated strains of *Vibrio fetus* is low or absent and that it rises under artificial cultivation to a certain maximum. This is not always true, however, as Table I shows. The vibrios of the three aborting cases are titrated against the serum of a rabbit treated repeatedly with cultures of Strain 67. In all cases a high agglutina-

[4] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

tion titer is demonstrated, although the strains are still in the early generations. The table also shows a general serological relationship among the strains tested.

The outcome of several tests of the blood serum of the three reported cases is summarized in Table II. Owing to a slight spontaneous agglutination in the controls, the high titers with Vibrio 356 will have to be reduced one or two degrees. The results, while not uniform, give encouragement that agglutination tests may be of use should this type of infectious abortion become more widespread. In

TABLE I.

Agglutination Tests with Serum from a Rabbit Immunized with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Serum dilutions.								Control.
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Vibrios in salt solution.
433	11th	C.*	C.	C.	C.	C.	C.	+++	+++	−
438	9th	"	"	"	"	"	"	C.	+++[1]	−
449	5th	"	"	"	"	"	"	"	+++	−
67	125th	"	"	"	"	"	"	"	+++	−

* C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and − no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

only one case (No. 449) was serum antedating the abortion on hand, and this was negative. We have, therefore, no way of knowing at present whether a high titer precedes abortion, as is almost regularly true in the presence of *Bacillus abortus*. By choosing some easily agglutinable strain which has been tested against normal sera it may be possible to detect this type of infection as readily as the other. All of the strains included in the table were tested against the serum of a normal heifer and no clumping was observed in dilutions ranging from 1:20 to 1:1,280.

TABLE II.

Agglutination Tests with Blood Serum of the Three Cases.

Case No.	Date of abortion.	Date of withdrawal of blood.	Vibrio used as antigen. Case No.	Transfer.	Date of test.	Serum dilutions. 1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Controls. Vibrios in salt solution.	Normal serum.
433	Oct. 24, 1919	Nov. 13, 1919	433	6th	Dec. 1, 1919	+++[1]	+++[1]	+++[1]	+++[1]	+++	++	−	−	−	−
		" 13, 1919	347	27th	" 1, 1919	+++[1]	+++[1]	+++	++	−	−	−	−	−	−
		Apr. 15, 1920	433	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	+++	++	+	±	±	−
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	+	+	−
438	Nov. 9, 1919	Nov. 13, 1919	438	4th	Dec. 1, 1919	++	+++[1]	+++[1]	+++[1]	+++	++	−	−	−	−
		" 13, 1919	347	27th	" 1, 1919	+++	+++	+++	++	−	−	−	−	−	−
		Apr. 15, 1920	449	16th	Apr. 19, 1920	C.	C.	+++[1]	+++	−	−	−	−	−	−
		" 15, 1920	356	32nd	" 19, 1920	"	+++[1]	+++[1]	+++[1]	+++[1]	+++	+++	++	+	−
449	Dec. 1, 1919	Nov. 25, 1919	449	7th	Jan. 31, 1920	−	−	−	−	−	−	−	−	−	−
		" 25, 1919	347	33rd	" 31, 1920	++	+	−	−	−	−	−	−	−	−
		Dec. 2, 1919	449	5th	" 19, 1920	++[1]	−	−	−	−	−	−	−	−	−
		" 2, 1919	347	33rd	" 31, 1920	+++	++	+	−	−	−	−	−	−	−
		Apr. 15, 1920	449	13th	Apr. 19, 1920	++	−	−	−	−	−	−	−	−	−
		" 15, 1920	356	32nd	" 19, 1920	+++[1]	+++	+++	+++	+++	++[1]	++	+	+	−

GENERAL SUMMARY.

The data bearing on these three cases are quite sufficient to rule out *Bacillus abortus* as the agent. Not only the cultures and guinea pig tests of fetal tissues and contents of the digestive tract, but also the agglutination and guinea pig tests of the milk, were negative. The same is true of the agglutination tests of the blood serum. Only in one case was the placenta obtained in part. The stained films and the sections from various regions showed no abortion bacilli. Guinea pig tests of placental tissue were negative for *Bacillus abortus*. On the other hand, minute organisms resembling vibrios were detected in the cytoplasm of endothelial cells within capillaries in the edematous subchorionic tissue. Subsequently the agglutination titer of the blood serum of one of these cases rose to a level indicating infection with *Bacillus abortus* during the second pregnancy.

The peculiar distribution of abortions due to *Vibrio fetus* among older cows and heifers in this herd, resulting at first in cases among older cows and latterly passing to young stock, may be explained by certain occurrences in the herd itself. It may be assumed that the infection was originally brought in by purchased cows. The young stock is kept segregated from these in a special barn, and when 6 months old it is pastured on outlying farms until returned in an advanced stage of pregnancy. The heifers during the first pregnancy were thus kept away from vibrio carriers until after the first calf was born.

In June and July, 1919, 55 older cows, purchased and native, were placed on the young stock pasture. The three cases of abortion in heifers due to *Vibrio fetus* occurred October 24, November 9, and December 2, 1919. The age and condition of the fetuses accord very well with the assumption that *Vibrio fetus* was introduced among the young stock in June or July of the same year.

The information gathered thus far concerning vibrionic abortion in this herd enables us to formulate a tentative hypothesis subject to modification with increasing knowledge of this type of infectious abortion. The infectious agent was probably introduced by purchased cows in 1917 or earlier. It gained a certain headway up to 1919, then the number of cases declined so that between May, 1919,

and May, 1920, only the above three cases in heifers, and one case of mixed infection with *Bacillus abortus* in an older cow, were detected. During the same period cases due to *Bacillus abortus* continued undiminished. The greater resistance of *Bacillus abortus* manifested in cultures as compared with *Vibrio fetus* is thus reflected in its behavior in nature. The temporary dying out of the infection indicates that natural immunization of a herd to *Vibrio fetus* proceeds quite rapidly. Another outbreak may be expected when the immunity of the herd has declined in the absence of the infecting agent and the latter is reintroduced from without, or it may reappear at any time when a vibrio of higher virulence is brought in.

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